REGULATORY PROTEINS AND GENES IN PLANT CELL DIVISION AND DIFFERENTIATION

BY

KERONG ZHANG

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STATEMENT

All the research reported in this thesis is original
and my own, except where due acknowledgment is made
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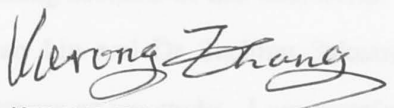

KERONG ZHANG

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ABSTRACT

An investigation has been made into the role of $p34^{cdc2}$-like protein kinase in; (1), the plant cell division cycle; (2), the developmental switch to cell differentiation, and (3), in the resumption of cell division by differentiated cells of tobacco pith. Genetic evidence in yeasts and biochemical evidence from animal cells has suggested that $p34^{cdc2}$ is a key cell cycle control protein.

For measurement of the level and activity of $p34^{cdc2}$-like protein kinase during the cell division cycle, suspension culture cells of *N. plumbaginifolia* were synchronised by various means. Cells were arrested predominantly in G1 phase when starved for nitrogen or phosphorous, preferentially in S phase by aphidicolin inhibition and predominantly in G2 phase by starvation for sucrose. The phase of arrest and subsequent synchronous progress through the cell cycle, on release of the block, was monitored by nuclear DNA content, nuclear DNA synthesis indicated by incorporation of BrdU into nuclear DNA, mitotic activity and increase in cell number. The level of $p34^{cdc2}$-like protein was measured using antibody specific for the 16 amino acid PSTAIR sequence that is perfectly conserved in all cdc2 homologues including those of plants. The level of $p34^{cdc2}$-like protein relative to other proteins was held relatively constant throughout the cell cycle in suspension culture cells that began a cell cycle in G1 phase, whether resuming after starvation for nitrogen or for phosphorous, and also in cells beginning in S phase after release from aphidicolin inhibition, as well as in cells beginning in G2 phase when resuming after starvation for sucrose. Data from nitrogen or phosphorous starved cells and from aphidicolin inhibited cells were considered more significant because a higher proportion of the population was in synchrony after these treatments. Although the enzyme protein level was relatively constant its $p34^{cdc2}$-like H1 histone kinase activity, measured after affinity purification of the enzyme by binding to $p13^{suc1}$, was found to peak on entry into M phase in suspension culture cells synchronised by all methods. An α-casein kinase activity could be detected in the affinity purified $p34^{cdc2}$-like protein kinase fraction in extracts from cells that were in transition from late G1 to S phase in synchronous division following starvation for nitrogen. The possibility that this

activity at G1/S derives from the presence of CDK protein kinase is discussed. The evidence from synchronous cultures is therefore consistent with involvement of $p34^{cdc2}$-like protein kinase in the transition between late G2 and M phases in the plant kingdom as in other eukaryotes, and it is possible that it is also involved in the transition from late G1 to S phase.

Consistent with the importance of protein phosphorylation at mitosis, which is implied by the peak of $p34^{cdc2}$-like kinase activity at this time, cells could be blocked in mitosis by disturbance of protein phosphorylation levels with okadaic acid. In the presence of this inhibitor of type 1 and type 2A protein phosphatases, cells progressed through G1 and S phases but arrested in early prophase. In *N. plumbaginifolia* cells inhibited by 12 μM okadaic acid arrest of the active population in early mitosis was indicated by G2 phase nuclear DNA content, partially condensed chromatin, persisting nucleolus and the occurrence of PPB microtubules. In frequency and timing the development of PPB microtubules was the same as in uninhibited control cells derived from the same synchronous culture. In 95% of inhibited cells PPB microtubules disassembled at the same time as in control cells, although nuclear events had not progressed beyond early prophase. Whereas in control cells, as in all other normal plant cells, PPB breakdown followed spindle formation and completion of chromosome condensation. The evidence that the persistence of normal PPB disassembly in 12 μM okadaic acid inhibition coincided with the persisting normal activation of $p34^{cdc2}$-like protein kinase, together with evidence for involvement of the kinase in cytoskeletal rearrangements in other cell types and the reported location of a PSTAIR-containing protein in the PPB, suggests the possibility that activation of $p34^{cdc2}$-like protein kinase is involved in initiating a signal for disassembly of PPB microtubules.

The level and activity of $p34^{cdc2}$-like protein kinase were four fold and three fold lower in short-term suspension culture cells of *N. plumbaginifolia*, which still retained both PPB microtubules and the ability to switch off cell division prior to differentiation into shoot and root tissues than in long-term suspension culture cells. The long-term cultured cells, which had four times as much $p34^{cdc2}$-like protein and three times as much of its activity, continued to activate $p34^{cdc2}$-like protein kinase preferentially at

mitosis but had lost the ability to switch off cell division and to differentiate into tissues and had lost PPB microtubules. It is possible that elevated $p34^{cdc2}$ kinase is incompatible with formation of a PPB. It is proposed that capacity to establish normal low levels of $p34^{cdc2}$-like protein kinase is a requirement for switching off cell division prior to differentiation in plant cells.

Effects of auxin and cytokinin class plant growth regulators (phytohormones) were investigated in terms of their effects on the accumulation and activation of $p34^{cdc2}$-like enzyme. The levels of $p34^{cdc2}$-like protein were investigated in suspension culture cells of *N. plumbaginifolia* and in excised pith parenchyma cells of tobacco Wisconsin 38 treated with auxins or cytokinins singly or in combination. Auxin phytohormones were the main inducers of $p34^{cdc2}$-like protein accumulation. The amount of $p34^{cdc2}$-like protein induced by 2,4-D in suspension culture cells and similarly in pith cells induced by NAA were more than tenfold higher than in uninduced cells. Treatment with kinetin-only or with BAP-only did not induce accumulation of $p34^{cdc2}$-like protein in suspension or pith cells, although, in these stem-derived cells, these cytokinin-class phytohormones were not inhibitory to its accumulation.

$p34^{cdc2}$-like H1 histone kinase activity measured in the same phytohormone treated cells showed that cytokinin was necessary for catalytic activation although cytokinins were unable alone to induce the enzyme protein. Pith parenchyma cells treated with both auxin and cytokinin showed high $p34^{cdc2}$-like protein kinase activity while treatment with NAA alone gave low activity although it induced accumulation of the enzyme protein. Similarly high H1 histone kinase activity of $p34^{cdc2}$-like protein could only be measured in cells from suspension culture in medium containing 2,4-D after additional supplementation with kinetin.

The activation of $p34^{cdc2}$-like kinase may have correlated with changes in phosphorylation state of the 34 kDa catalytic subunit because a slower-migrating band of $p34^{cdc2}$-like protein, which is probably a phosphorylated form of $p34^{cdc2}$ that has been detected in oocytes, animal cell lines and in *Chlamydomonas*, was found in plant cell extracts. The possible relationship between the slower-migrating band and the regulation of activity of $p34^{cdc2}$-like protein kinase in tobacco cells is discussed. Evidence that

changes in phosphorylation can be involved in the activation of plant $p34^{cdc2}$ was provided by the *in vitro* activation of the enzyme by incubation with acid phosphatase when the enzyme was drived from suspension culture cells that were arrested in G2 phase by lack of exogenous cytokinin. The possibility is discussed that partial dephosphorylation by removal of phosphates from tyrosine 15 and threonine 14 is involved in the activation of plant $p34^{cdc2}$.

The possible differential requirement of auxin and cytokinin at key cell cycle control points was investigated by incubating suspension culture cells of *N. plumbaginifolia* that require both auxin and cytokinin in medium with 2,4-D-only or kinetin-only and then investigating their point of arrest and the pattern of their resumed cycling. Cells were arrested in both G1 and G2 phases in suspension culture lacking auxin and containing kinetin only, while cells were arrested exclusively in G2 phase in suspension culture lacking kinetin and containing 2,4-D only. This arrest pattern was indicated by nuclear DNA content and by the kinetics with which the population activated $p34^{cdc2}$-like protein kinase and progressed through mitosis to cell number increase. An arrest point in late G2 prior to the activation of $p34^{cdc2}$-like protein kinase in cells lacking cytokinin, from which point early activation of the enzyme occurs on resupply of cytokinin, indicates that an early effect of cytokinin in cells in which it stimulates division may be activation of the key cell cycle regulating kinase.

These findings suggest that auxin stimulates the synthesis or accumulation of $p34^{cdc2}$-like protein kinase and that cytokinin is additionally required for the activation of its $p34^{cdc2}$-like protein kinase catalytic activity. Auxin (2,4-D) is required for the transition from late G1 to S phase and both auxin and cytokinin (kinetin) are required for the transition between late G2 and M phases in suspension culture cells of *N. plumbaginifolia*. It is proposed that the induced accumulation and activation of $p34^{cdc2}$-like protein kinase is involved in switching on cell division in hormone-stimulated differentiated cells, as may occur naturally in lateral meristem activation, in secondary thickening and in wound response and that the mechanisms by which auxin and cytokinin phytohormones act on cell division and dedifferentiation in plants at least partly involve changes of level and activity of $p34^{cdc2}$-like protein kinase.

LIST OF ABBREVIATIONS

cAMP	cyclic adenosine-3-5-monophosphate
ATP	adenosine triphosphate
ATP	adenosine triphosphate
6-BA	6-benzyladenine
BAP	6-(benzyl amino) purine
BCIP	5-bromo-4-chloro-3-indolephosphate p-toluidine salt
bp	base pair
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
cdc	cell division cycle
cdc2	cell division cycle gene identified in fission yeastby mutation and arbitarily numbered 2. The equivalent gene in budding yeast is designated CDC28 or *CDC28*; mutant form of these genes are designated *cdc2* or *cdc2*$^{-}$, and *cdc28* respectively, following the conventions of investigators of the two organisms. In this thesis where the generic type of gene is referred to in other eukaryotes (rather than the specific gene in yeast), the simpler designation cdc2 is used. Throughout, the protein products are referred to as cdc2 protein or cdc28 protein. The same conventions, with respect to use of upper and lower case and italics, are followed with other cell cycle genes.
CDK	cyclin dependent kinase
2,4-D	2,4 dichlorophenoxyacetic acid
DAPI	4', 6'-diamidineo-2-phenylindole
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediamine N, N, N', N'-tetraacetic acid
EGTA	ethyleneglycol-bis-(β-aminoethyl ether) N, N, N', N'-tetraacetic acid
G0	developmentally programmed cessation of division that can be reversed only slowly by resumption of protein synthesis after hormone stimulation
G1	gap 1, the period between cell division and S phase
G2	gap 2, the period between S phase and nuclear division
HeLa	line of human neoplastic (cervical) epithelial cells
IAA	indole-3-acetic acid
IPA	N^6-(2-isopentenyl) adenosine
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase
kDa	kilodalton

Kinetin	6-furfurylaminopurine
LB medium	Luria-Bertani medium
M phase	mitotic phase, nuclear division in somatic cells
MAP	microtubule associated protein
MES	(N-morpholino) ethanesulfonic acid
MS medium	Murashige-Skoog medium
NAA	napthyl acatic acid
NBT	nitroblue tetrazolium chloride
NP 40	NONIDET P-40, nonionic detergent
$p34^{cdc2}$	34 kDa protein encoded by the *cdc2* gene of fission yeast; $p34^{CDC28}$ homologous protein encoded by the CDC28 gene of budding yeast
PBS	phosphate buffered saline (described in 2.2.3)
PCR	polymerase chain phosphatase
PGP	phragmoplast
PIPES	piperazine-N, N'-bis (2-ethanesulfonic acid)
PM5E	microtubule stabilizing buffer
PMSF	phenylmethysulfonyl fluoride
PPB	preprophase band
PSTAIR	EGVPSTAIREISLLKE amino acid residues
PTP	protein tyrosine phosphatase
RIPA buffer	multi-component protein extraction buffer (described in 2.2.5.1)
RNA	ribonucleic acid
S phase	phase of synthesis of nuclear DNA
SDS	sodium dodecyl sulfate
TB	transfer buffer
TBS	tris buffered saline
TCA	trichloroacetic acid
TEMED	N, N, N', N'-tetramethyl ethylene diamine
tris	tris (hydroxymethyl)-aminomethane
Tween 20	polyoxyethylenesorbitan monolaurate
UV	ultra violet

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CHAPTER 1

INTRODUCTION

The cell can be considered a fundamental unit of both structure and function in all living things. All forms of life, from prokaryotes to eukaryotes, are composed of cells and their secretions. However, cells arise only by division of preexisting cells. The process involves the duplication of cellular components and their partition into daughter cells and this can be termed the cell cycle. Death results if the cycle fails in growing cells and cancer can result if it is improperly regulated in mature cells, which must often switch from proliferation to differentiation. This switch allows the performance of specialised functions, which involve changes in cell structure and biochemistry that are incompatible with continued division.

Most cell cycles can be divided (Howard and Pelc, 1953) into phases of; G1 (gap before DNA synthesis), S (DNA synthesis), G2 (gap after DNA synthesis), and M (mitosis) and in most cell cycles a final cytokinetic phase occurs in which the cytoplasm separates into two daughter cells.

Since the 1970's, the mechanisms by which events in the cell cycle phases are regulated have come under genetical and biochemical investigation.

1.1 Genetic analysis of cell division cycle in yeasts

Yeast is a simple eukaryotic organism which is an ideal subject for the investigation of complex cellular processes because it has a very small nuclear genome, which in budding yeast is not quite four times the size of that of the bacterium *Escherichia coli*. Moreover, yeast is easily mutated to obtain modified proteins with altered thermolability. This form of mutation has been most widely used for the analysis of the cell cycle since it allows propagation of the experimental organism at the permissive temperature and, by shift to a restrictive temperature that inactivates only the protein encoded by the mutated gene, it allows the identification of events that require the normal protein. Most of these

mutants become blocked at a specific stage of the cell division cycle (*cdc*) when incubated at the restrictive temperature. These *cdc* mutations have led to the formulation of a model of the cell cycle as comprising events organised in a dependent sequence, each requiring the completion of the preceding event (Hartwell et al ., 1973, 1974).

Hartwell and his colleagues began genetic analysis of budding yeast *Saccharomyces cerevisiae* using *cdc* mutants (Hartwell et al., 1973) and to date about 150 such mutants have been isolated. The analysis of complementation ordered these recessive mutations into 32 groups, and tetrad analysis revealed that each of these groups was defined as a single nuclear gene (Hartwell et al., 1973). In 1974, they suggested that the cell cycle is ordered in dependent pathways within which a later event is dependent upon the completion of an earlier event. The phenotypes of the *cdc* mutants suggested that separate gene functions were required at; "START" in late G1 phase; initiation of DNA synthesis; medial nuclear division; late nuclear division; cytokinesis; and cell separation. The START of division in late G1 is a target for signals mediating cell division cycle control. Specifically, it was found that *cdc28* mutants are blocked at START and the *CDC28* gene product is required for a cell to pass through START (Hartwell et al., 1974; Reed 1980). Once a cell has passed START it is committed to pass through S, G2, M and cytokinesis phases even if no further net cell growth is possible and even if the presence of a pheremones from a cell of opposite mating type signals an opportunity for mating. Prior to START, division is not committed and cells will only execute START if their growth rate and size are adequate and if mating pheremones are absent.

In the fission yeast, *Schizosaccharomyces pombe*, 27 recessive temperature sensitive mutants, which were unable to complete the cell division cycle at the restrictive temperature, were isolated by Nurse and his colleagues (Nurse et al., 1976). These mutants defined 14 unlinked genes which are involved in DNA synthesis (*cdc10*), nuclear division (*cdc1, cdc2, cdc5, cdc6* and *cdc13*) and early cell plate formation (*cdc3, cdc4, cdc8* and *cdc12*). Specifically, when *cdc2* mutants were transferred from normal culture temperature to the restrictive temperature, 35°C, they synthesised DNA for a short time and then stopped DNA synthesis, and nuclear division was inhibited (Nurse et al., 1976).

Further evidence showed that *cdc2* gene function is the major rate-limiting step determining the time of mitosis (Nurse and Thuriaux, 1980). In 1981, Nurse and his colleagues found a START control point in *S. pombe*, which is located in late G1 phase and is homologous with START in budding yeast *S. cerevisiae*. Mutational analysis revealed that the *cdc2* gene is required for completion of start control. They therefore proposed that *cdc2* is a cell cycle control gene which acts at two separate points in the cell cycle, being required in G1 for commitment to cell division and in G2 for the control of mitosis (Nurse and Bissett, 1981). The data of Piggot et al (1982) indicated that *CDC28* in budding yeast had a similar dual function.

1.2 *cdc2/CDC28* genes in yeasts

Based on this genetic analysis, *cdc2* was deduced to be an essential control gene at the transition from G1 to S and the transition between G2 and M phases in fission yeast (Nurse and Bissett, 1981). Similarly, the *CDC28* gene was shown to control START in G1 phase in budding yeast (Hartwell et al., 1974; Reed, 1980), but its evident function at mitotic phase was not recognised in Reed's laboratory until 1990 (Reed and Wittenberg, 1990) although some *cdc28* mutants arrest at G2 phase just before mitosis (Piggot et al., 1982) and, very significantly, Beach, Durkacz and Nurse (1982) had found that the *CDC28* gene from budding yeast complemented the fission yeast *cdc2* mutation that blocked both START and mitosis. Beach et al (1982) concluded that the *cdc2* gene and *CDC28* gene perform homologous cell cycle control functions in the two organisms.

Biochemical evidence further confirmed the control function of the *cdc2/CDC28* gene in the cell division cycle. The c*dc2/CDC28* genes (occupying about 1.69 kb DNA) were isolated and sequenced (Nasmyth and Reed, 1980; Beach et al., 1982; Hindley and Phear, 1984). The *S. pombe cdc2* DNA is split by four introns. The *CDC28* DNA fragment does not contain introns and has a 298 codon open reading frame. The predicted primary structure of *cdc2/CDC28* genes contains the consensus sequence for ATP-binding and phosphorylation-acceptor sites of protein kinases such as bovine cAMP-dependent protein kinase and the *src* family of viral oncogene products (Lorincz

and Reed, 1984; Hindley and Phear, 1984). The products of the *cdc2* and *CDC28* genes are both 34 kDa serine-threonine protein kinases. Protein kinase activity attributed to the *cdc2* gene product $p34^{cdc2}$ was high in proliferating fission yeast with a peak during M phase but fell to a low level during interphase and in cells withdrawn from the cell cycle (Simanis and Nurse, 1986; Booher et al., 1989). The cdc28 protein kinase is extractable in catalytically active form from budding yeast cells in transition from G1 to S phase (Wittenberg and Reed, 1988) and in the transition between G2 and M phase (Reed and Wittenberg, 1990).

Homologues of the *cdc2* gene and its product have been found in species including higher eukaryotes, as discussed later in this Chapter. The *cdc2* gene is therefore considered a universal cell division cycle gene of eukaryotes (Nurse, 1990).

In addition, genes that are involved in regulation of the *cdc2* gene product have also been discovered to have equivalents in high eukaryotic cells.

1.3 *cdc* genes which regulate *cdc2* gene products and their homologues in yeasts and higher eukaryotes

Genetic and biochemical evidence shows that the *cdc2* gene product is regulated by products of the *cdc25*, *nim1* and *wee1/mik1* genes and it is physically associated *in vivo* with *suc1* and cyclin proteins.

1.3.1 *cdc25* and homologues

The *cdc25* gene was identified by mutational analysis in *S. pombe*. Cells containing either of the mutant alleles *cdc25.22* or *cdc25*.M51 were able to undergo mitosis and cell division at 25°C, but when shifted to 35°C they accumulated in G2 phase with a single nucleus. This evidence suggested that $cdc25^+$ is involved in mitotic control in *S. pombe* (Fantes, 1979). The $cdc25^+$ gene product counteracts the mitotic inhibition exerted by $wee1^+$, and a balance of $wee1^+$ and $cdc25^+$ activities regulates mitotic timing in fission yeast (Russell and Nurse, 1986). The $cdc25^+$ gene was isolated and sequenced and was shown to encode a protein of 80 kDa (Russell and Nurse, 1986). The cdc25 protein is

periodically accumulated in the yeast cell cycle but more continuously in animal cells and is catalyses a rate-limiting processes regulating the initiation of mitosis (Moreno et al., 1990; Giard et al., 1992). The fact that *S. pombe* mutants that lacked active cdc25 protein accumulated phosphorylated tyrosine in cdc2 protein (Gould and Nurse, 1989) and the fact that these mutants could be rescued by introduction of the gene for a human tyrosine phosphatase (Gould et al., 1990) indirectly indicated that cdc25 protein is a tyrosine phosphatase.

Homologues of *cdc25* have been found in higher eukaryotic cells. For example, the homologues of *cdc25*, Xcdc25 in *Xenopus*, $CDC25^{Mm}$ in mouse, twine and string (stg) in *Drosophila* and cdc25A, cdc25B and cdc25C in human cells have been detected by genetic complementation analysis or by probing for conserved sequences using the polymerase chain reaction (PCR) (Edgar and O'Farrell, 1989; Sadhu et al., 1990; Galaktionov and Beach, 1991; Kumagai and Dunphy, 1991; Alphey et al., 1992). The cdc25 genes from *Drosophila* and human cells rescue fission yeast *cdc25* mutations (Edgar and O'Farrell, 1989; Sadhu et al., 1990; Galaktionov and Beach, 1991; Alphey et al., 1992). This may not be a rigorous test for close homology given that an unrelated tyrosine phosphatase can complement (Gould et al., 1990), which may indicate that rather few proteins in *S. pombe* have phosphorylated tyrosine and that a nonspecific phosphatase may therefore serve to maintain cell viability. The predicted cdc25 products in *Drosophila* and human are 53-67 kDa, and have sequence similarity, in a 20 kDa catalytic domain located near the C-terminus, with the equivalent region of yeast cdc25 protein (Edgar and O'Farrell, 1989; Sadhu et al., 1990; Galaktionov and Beach, 1991; Alphey et al., 1992). It is evident that homologues of *cdc25* can be found in a wide range of eukaryotic species and therefore probable that cdc25 gene is a universally conserved M phase inducer.

It was suggested that the cdc25 protein is a distantly related member of the known PTPases (protein tyrosine phosphatases) because the cdc25 protein consensus sequences derived from the *cdc25* genes of fission and budding yeasts, *Drosophila* and human contain a small degree of homology to the conserved active site of PTPases (IVXHCXXXXX R) which is called the HC motif (Strausfeld et al., 1991). Like cdc25

proteins in yeast, the products of cdc25 homologues share a degree of similarity with a newly identified phosphatase that can dephosphorylate both threonine and tyrosine residues (Kumagai and Dunphy, 1991; Millar and Russell, 1992). More significantly, the bacterially-produced *Drosophila* cdc25 protein can catalyse the dephosphorylation of several phosphatase substrates, including p-nitrophenyl phosphate and two distinct tyrosine-phosphorylated peptides (Dunphy and Kumagai, 1991). The phosphatase activity of the *Drosophila* cdc25 protein displays many of the properties of the known tyrosine phosphatases. For example, the hydrolytic activity requires dithiothreitol and does not depend on divalent cations (Dunphy and Kumagai, 1991). It is significant that cdc25 phosphatase can remove phosphate from both threonine (Thr) and tyrosine (Tyr). In this respect it is an unsual phosphatase but this property is relevant to the activation of $p34^{cdc2}$ in higher eukaryotes by dephosphorylation of both Thr 14 and Tyr 15.

The level of cdc25 mRNA and its product $p80^{cdc25}$ in *Drosophila* (Edgar and O'Farrell, 1989) and in HeLa cells (Sadhu et al., 1990) oscillates through the cell reaching a maximum at cell division. Although the cdc25 protein of *Xenopus* does not oscillate in level during the cell cycle it is directly associated with cdc2-cyclin B complex in a cell cycle-dependent manner reaching a peaking at M phase, indicating that cdc25 triggers cdc2 activation by a periodic physical association with the cdc2-cyclin B complex (Jessus and Beach, 1992). Starfish and human homologues of the cdc25 protein promote the maximal activation of doubly phosphorylated $p34^{cdc2}$-cyclin (Galaktionov and Beach, 1991; Gautier et al., 1991; Strausfeld et al., 1991). It is now considered that the products of *cdc25* homologues in higher eukaryotic cells, like cdc25 protein in yeast (Moreno and Nurse, 1991), function in the dephosphorylation of tyrosine 15 (Tyr 15) in $p34^{cdc2}$ homologues (Galaktionov and Beach, 1991; Gautier et al., 1991).

1.3.2 *wee1*, *mik1* and homologues

In *S. pombe*, it has been noted that growing cells tend to maintain a constant size at division (Mitchison, 1971). This means that there is coordination between cellular growth and cell division, and probably this coordination results from division occurring when the cell has grown to a particular size. In 1978, Thuriaux and his colleagues

isolated twenty-five mutants which had the same growth rate as the wild-type but divided at half of the wild-type size, at a cell length of between 7.7 and 8.2 μm. These mutants were defined as *wee*1 and *wee*2 genes, both of which are involved in controlling the initiation of mitosis when the cell attains a critical size (Thuriaux et al ., 1978). In 1980, Nurse and Thuriaux further isolated fifty-two *wee* mutants which undergo mitosis and cell division at a reduced size compared with the wild-type. These mutations were also found to be in the *wee*1 or *wee2* genes. The *wee2* gene was recognised, by mapping to the *cdc2* gene, as a mutant form of the *cdc2* gene while *wee1*$^+$ mapped separately and was deduced to code for a negative element or inhibitor in mitotic control (Nurse and Thuriaux, 1980). In addition, the *wee1*$^+$ function was found to be dosage-dependent by analysis of the cell division size phenotype of *wee1*$^+$/*wee1*$^-$ diploids (Nurse and Thuriaux, 1980). The *wee1* gene was cloned and its sequence revealed a 2.7 kb open reading frame encoding a 107 kDa protein (Russell and Nurse, 1987a). This *wee1*$^+$ gene product is representative of a novel class of protein kinases that phosphorylates both threonine and tyrosine residues (Featherstone and Russell, 1991; Parker et al., 1992). It is possible that wee1 protein kinase acts on the threonine and tyrosine residues of p34^{cdc2}, negatively regulating the p34^{cdc2} protein kinase activity.

The question of why mitosis was merely advanced rather than completely unregulated in cells lacking a function of wee-1 protein was investigated in Beach's laboratory and it was discovered that a second gene, *mik1*, could be cloned by its capacity to antagonise the catastrophic effects of excessive *cdc25*$^+$ gene expression in *wee-1*$^-$ mutant cells (Lundgren et al., 1991). The *mik1* gene sequence indicates a considerable similarity of its product to wee-1 protein kinase and the circumstance in which it was cloned indicates that it has a similar function *in vivo*, however the mik1 enzyme activity has so far not been biochemically characterised.

A few possible homologues of wee-1 have been found to be present in higher eukaryotes. For example, in human cells, a possible human homologue of *wee-1* has been found by its ability, when overexpressed in HeLa cells, to block cell division (McGowan and Russell, 1993). Purified human wee-1 protein phosphorylates p34^{cdc2} exclusively on Tyr 15, not on Thr 14, and specifically phosphorylates synthetic peptides

at a site equivalent to Tyr 15 (McGowan and Russell, 1993). It is possible that the human homologue of wee-1 protein kinase regulates the activity of the $p34^{cdc2}$ homologue in human cells by phosphorylating tyrosine (McGowan and Russell, 1993).

1.3.3 *nim1/cdr1*

In 1987, Russell and Nurse found another gene, *nim1*$^+$ (new inducer of mitosis), which is involved in the regulation of mitotic control. The gene was detected by the transformation and complementation of a temperature sensitive *cdc25* mutant with an *S. pombe* gene bank. Increased *nim1*$^+$ expression causes *cdc25*$^+$ cells to divide at about half the size of the wild-type because of their earlier initiation of mitosis. The *nim1*$^+$ gene product induces mitosis in a dose dependent manner. In addition, it was found that *nim1*$^+$ functions as a negative regulator of *wee1*$^+$ through an analysis of function of *nim1*$^+$ in *wee1*$^+$ and *wee1*$^-$ mutants (Russell and Nurse, 1987b), which showed that the *nim1* gene has no effect in cells lacking *wee-1*$^+$. The *nim1* gene was cloned, sequenced and an open reading frame was recognised with a predicted product of 50 kDa molecular weight (Russell and Nurse, 1987b). More recently it has been recognised that *nim1* gene as cloned initially was slightly truncated and the full length gene has been named *cdr1* (Feilotter et al., 1991). The nim1/cdr1 protein has been shown to be 20%-35% identical to the catalytic domain of a family of protein kinases, which includes cAMP-dependent protein kinase and the *v-src* oncogene product, and it was suggested that the *nim1* gene product is a protein kinase because it contains an ATP binding site which is a characteristic of protein kinase (Russell and Nurse, 1987b). The fact that the *nim1*$^+$ gene product inhibits the function of the *wee1*$^+$ gene product and that increased *nim1*$^+$ expression suppresses *cdc25* mutations shows that nim1 protein kinase functions as a negative regulator of *wee1*$^+$ gene products in fission yeast (Russell and Nurse, 1987b).

Identification of the control elements described above therefore suggests that the regulation of $p34^{cdc2}$ at the transition from late G2 to M phase involves a cascade containing the products of *cdc25*, *wee1* and *nim1* genes. Further aspects of $p34^{cdc2}$ control were indicated by the discovery that two kinds of genes encode proteins that

physically associate with the *cdc2* gene product. These are *suc1/CKS* genes and cyclin genes.

1.3.4 *suc1* and homologues

The *suc1* gene was found by screening a cDNA library in certain *cdc2* mutant cells for capacity to allow colony formation at the restrictive temperature. An additional copy of the *suc1* gene could restore function to particular *cdc2* mutants. The effect is therefore allele specific and consequently provided an early indication that the two proteins $p13^{suc1}$ and $p34^{cdc2}$ interact physically. Strong overexpression of the *suc1* gene when driven by an active promoter, lead to a delay in mitosis (Hayles et al., 1986 a, 1986 b). The *suc1* gene product is essential for cell viability because cells which lose the *suc1* gene product either become elongated, indicating some block in the cell cycle, or are impaired in cellular growth. These results suggest that the suc1 protein, in association with the cdc2 protein, is necessary for the cell cycle and cellular growth (Hayles et al., 1986 a, 1986 b; Brizuela et al., 1987; Hindley et al., 1987). A homologue of the *suc1* gene , named *CKS1*, was found in *S. cerevisiae* by the same strategy of rescuing a temperature-sensitive *CDC28* mutation. The *CKS1* gene functions at START and its product is associated with the *CDC28* gene (Hadwiger et al., 1989a).

The *suc1*$^+$ gene is 1083 bp long and encodes a protein of 13 kDa ($p13^{suc1}$) (Hindley et al., 1987). The *CKS1* gene product has a high similarity to the *suc1* gene product but it is a little larger due to the presence of a tail with a high proportion of glutamine residues at the carboxyl end of the protein. The level of $p13^{suc1}$ is unaltered during the cell cycle and also during entry into stationary phase as determined by analysis using antibody against $p13^{suc1}$. In *S. pombe* lysates, the $p13^{suc1}$ forms a complex with $p34^{cdc2}$, and about 5% of the total cellular $p34^{cdc2}$ is associated with $p13^{suc1}$ and this fraction of $p34^{cdc2}$ has protein kinase activity (Brizuela et al., 1987). However, $p13^{suc1}$ is not a substrate of the $p34^{cdc2}$ protein kinase. Similarly, in *S. cerevisiae*, the 18 kDa CKS1 protein is physically associated with active forms of the CDC28 protein kinase (Hadwiger et al., 1989a). It has been proposed that $p13^{suc1}$ acts as a regulatory component of $p34^{cdc2}$ that facilitates interaction with other proteins (Hayles et al.,

1986b; Booher et al., 1989; Dunphy and Newport, 1989). Deletion of the *suc1* gene causes arrest at anaphase of mitosis and persistence of the active form of $p34^{cdc2}$ (Moreno et al., 1989) it is therefore implicated in the inactivation of $p34^{cdc2}$ that is necessary for anaphase.

1.3.5 Cyclins and cdc2 control

A current hypothesis is that cyclin proteins are essential for directing the catalytic activity of the $p34^{cdc2}$ protein kinase subunit to specific substrate proteins (Hunt, 1989; Pines and Hunter, 1991a). Cyclins were first detected in higher eukaryote cells by their cyclical pattern of synthesis in the mitotic cycle (Evans et al., 1983). Their complexing with the cdc2 catalytic subunit in cells from diverse taxa, and in animal cells their complexing with CDK variants, indicates a universal but complex cell division control mechanism.

The first mutation of a cyclin protein was isolated in fission yeast where the *cdc13* mutation blocked progress through the cell cycle in mid-mitosis with three over-condensed chromosomes clearly visible (Nurse et al., 1976; Nasmyth and Nurse, 1981). Cells were arrested in interphase when a major part of the $cdc13^{+}$ gene was deleted, but partial loss of gene activity lead to cells containing condensed chromosomes, aberrant septa and a microtubular cytoskeleton with was characteristics of both G2 and M phases. This evidence shows that $cdc13^{+}$ gene function is required for control of the G2 to M transition (Hagan et al., 1988). Subsequent sequence comparison with animal cyclins that peak in abundance at the time of mitosis has shown that *cdc13* encodes a mitotic cyclin (cyclin B). This class of protein has been more recently detected in the budding yeast by seeking proteins that could correct a defect in the mitotic function of the *CDC28* mutation *cdc28-4*. By this means, *CLB1*, *CLB2*, and *CLB4* genes were identified in *S. cerevisiae*. In addition the polymerase chain reaction, using primers based on cyclin gene sequences, revealed a further cyclin gene *CLB3*. These four genes were sequenced, and the amino acid sequences predicted in their products are similar to the amino acid sequence of the G2 cyclin cdc13 (Surana et al., 1991).

A different class of cyclins that is necessary in G1 phase was later recognised as necessary at the earlier cell cycle control point of START. Genes encoding these G1 cyclins were first identified by mutation before the class of cyclin proteins was biochemically characterised. The mutation *WHI1-1* in *S. cerevisiae* advances the time of START and *WHI1-1* mutant cells can initiate a new cell cycle at half the volume of wild-type cells (Sudbery et al, 1980). Nash and his colleagues provided evidence that *WHI1-1* was a dominant mutation that reduced cell volume by allowing cells to commit to division at an abnormally small size, shortening the G1 phase of the cell cycle (Nash et al., 1988). A mutation in the same gene, called *DAF1*, was independently isolated on the basis of its resistance to the cell cycle arrest in late G1 that normally occurs in the presence of peptide mating pheromones and this correlates with increased propensity to execute START (Cross, 1988). At the time of this earlier work cyclins had not been characterised and sequences of the *WHI-1/DAF1* gene had not been obtained therefore the identity of *WHI-1* with *DAF1* and their similarity with cyclins could not be recognised. However a later screen for dosage dependent suppressors of mutations that affected the START function of *CDC28* identified two other G1 cyclin genes, *CLN1* and *CLN2*. The mutations that identified *CLN1* and *CLN2* were found to have removed a proteolysis site in the encoded cyclin protein therefore increasing their stability and resulting in a dominant phenotype due to elevated cyclin levels that advanced the G1 to S phase transition in cycling cells and impaired the ability of cells to arrest in G1 phase in response to slow growth, small size or presence mating pheremone (Hadwiger et al., 1989b). Sequence analysis of the *CLN* genes revealed an homology with mitotic cyclins (Hadwiger et al., 1989b). Therefore *CLN1, CLN2* and *CLN3* (*WHI1/DAF1*) are G1 cyclin genes that are required in the G1 to S transition. Presence of at least one of these genes in functional form is required for progression through G1 to S phase, and deletion of all three leads to G1 arrest (Richardson et al., 1989; Reed, 1991). In fission yeast, a G1 cyclin gene called *puc1*$^{+}$ was found by screening for its ability to complement lack of *CLN* genes in *S. cerevisiae* (Forsburg and Nurse, 1991). The G1 cyclin genes and the mitotic cyclin genes (*cdc13* cyclin) both function in the regulation of the cell cycle by association of the proteins that they encode with the product of the *cdc2/CDC28* gene.

The proteins cln1, cln2 and cln3 (daf1/whi1), which are encoded by *CLN1, CLN2* and *CLN3* genes and associate with $p34^{CDC28}$, contain a protease sensitive region (named PEST sequence) that is rich in proline, glutamate, serine, threonine, and aspartate, and flanked by basic amino acids. Both cln1 and cln2 are similar in the region of the PEST sequence although the carboxyl-terminal domains of cln1 and cln2 share little primary structure homology (Hadwiger et al., 1989b). However, cln3 is much less closely related with cln1 and cln2 at the level of primary structure (Hadwiger et al., 1989b). Cln2, a 62 kd polypeptide, accumulated periodically, peaked during G1, declined rapidly afterwards, and was rapidly lost following the exposure of cells to mating pheromone which switches cells away from cell division and arrests them prior to START (Wittenberg et al., 1990). The cln2 polypeptide interacted with $p34^{CDC28}$ to form an active protein kinase complex that could be recovered by antibody against the cln2 polypeptide, which resulted in coimmunoprecipitation of $p34^{CDC28}$ that could phosphorylate H1 histone (Richardson et al., 1989; Wittenberg et al., 1990). The cln3 protein was found to be a very unstable, protein in low abundance (Tyers et al., 1992) and, like cln1 and cln2, associated with $p34^{CDC28}$ to form an active kinase complex. This complex phosphorylated cln3 itself and a coprecipitated substrate of 45 kDa (Tyers et al., 1992). The timing of cell cycle initiation in mother and daughter cells was governed by different G1 cyclins. Cln1 and cln2 were critical for START timing in daughter cells (newly released buds), however, cln3 was required for a normal duration of G1 in mother cells which had previously produced a bud (Lew et al., 1992; Linskens et al., 1993).

The *cdc13* gene product of fission yeast is a 56 kDa protein ($p56^{cdc13}$) but is sometimes also called $p63^{cdc13}$. From an analysis of allele-specific interactions between *cdc2* and *cdc13* mutants and from the fact that $cdc2^{+}$ over-expression suppresses the partial function mutant *cdc13-117,* Brizuela et al (1987) suggested that *cdc2* gene product and *cdc13* gene product interact closely. The sequence of $p56^{cdc13}$ has approximately a 50% identity with animal B cyclins (Hagan et al., 1988; Solomon et al., 1988). $p56^{cdc13}$ is present before the initiation of mitosis and is retained to the time the cells have undergone chromosome condensation, but as in the case of cyclin B in oocytes

its levels are reduced dramatically at the inactivation of $p34^{cdc2}$ that is necessary for the initiation of anaphase (Moreno et al., 1989).

The availability of cloned cyclin genes in yeasts allows a test of which cycle events are responsive to the level of cyclin protein and which may simply require the presence of cyclin. Expression of additional copies of the *cdc13* gene in *S. pombe* has shown that the timing of mitosis is unaffected, therefore the level of cyclin B does not affect the time at which $p34^{cdc2}$ is activated at mitosis. This timing is determined by the timing of changes in phosphorylation at Tyr 15 and Thr 14. Constant expression of additional G1 cyclin genes, or of genes that encode more stable G1 cyclin proteins results in a high level of G1 cyclin protein and an earlier execution of START in budding yeast. This suggests that the amount of G1 cyclins is limiting in G1 phase for the activation of $p34^{CDC28}$. G1 and G2 cyclins therefore both act through influencing $p34^{cdc2/CDC28}$ but probably do so in different ways.

The homologues of mitotic cyclins, like the cdc13 protein (Booher and Beach, 1988; Nash et al., 1988), which are involved in the control of the G2 to M phase transition of the cell cycle, have been studied in a number of oocytes and in mammalian cell lines. The two cyclins, known as cyclin A and B, have been detected in the clam *S. solidissima*. Their sequences show encoded products of 420 amino acid with a similar region of about 150 amino acids in the middle but difference at either end (Swenson et al., 1986). The B-type cyclin has been cloned from sea urchins (Pines and Hunt, 1987), frogs (Minshull et al., 1989), *Drosophila* (Lehner and O'Farrell, 1989; Whitfield et al., 1989) and humans (Pines and Hunter, 1989; Wang et al., 1990). Measurement of mRNA level and the ratio of incorporation of radioactive amino acids into cyclin A and the cyclin B indicate that these proteins are synthesised periodically during the cell cycle, accumulating during early and late G2 phase respectively when they reach 20 times higher levels than in G1 phase and then abruptly disappearing at mid mitosis (Lehner and O'Farrell, 1989; Minshull et al., 1989; Murray and Kirschner, 1989; Murray et al., 1989; Pines and Hunt, 1987, 1989, 1990a; Wang et al., 1990; Walker and Maller, 1991). The homologues of $p34^{cdc2}$ protein kinase in oocytes and human cells only become activated with respect to mitotic substrates when in complex with cyclin B (Chapter 1.4.3) and

$p34^{cdc2}$ is inactivated in anaphase when the complex is disassembled by cyclin proteolysis (Draetta et al., 1989; Gautier and Maller, 1991; Meijer et al., 1989; Roy et al., 1991; Solomon et al., 1990; Steinmann et al., 1991).

The cyclin family is becoming difficult to characterise by amino acid sequence in higher eukaryotes, where sequence similarity reveals a large number of proteins that may have some cyclin like properties. Even cyclins that are genetically demonstrated to have clear cell cycle function have only limited sequence homology which is restricted to the central 150 amino acid "cyclin box" region. Some G1 cyclins have been detected in higher eukaryotes. In *Drosophila*, a G1 cyclin named cyclin C was cloned and sequenced by rescuing *S. cerevisiae CLN* mutants with *Drosophila* cDNA clones. The *Drosophila* cyclin C is highly homologous (72% identity) to human G1 cyclin C (Leopold and O'Farrell, 1991). In mice, three G1 cyclins (CYL1, CYL2 and CYL3) / $p36^{CYL}$ were isolated, and two of these are regulated by colony-stimulating factor (CSF-1) during the G1 phase of the mouse macrophage cell cycle. During G1 phase, $p36^{CYL}$ is phosphorylated and associates with a polypeptide antigenically related to $p34^{cdc2}$. It was suggested that CYL may be required during S phase commitment because $p36^{CYL}$ synthesis is concurrent with the transition from G1 to S phase, and its phosphorylation and transient binding to a cdc2-related polypeptide also occur in transition from G1 to S phase (Matsushime et al., 1991). In human cells, G1 cyclins were also detected besides the mitotic cyclins. Cyclin C, D, and E genes have been identified by isolating cDNA clones derived from human mRNA which are able to substitute for G1 cyclin genes in *S. cerevisiae* (Lew et al., 1991). Cyclin D1, which was prepared from a human glioblastoma cell line, could rescue a budding yeast strain that lacked cln1 and cln2 and was conditionally deficient for cln3 function in *S. cerevisiae* (Xiong et al., 1991). These human G1 cyclins can rescue the G1 cyclin mutations in *S. cerevisiae* and like them are associated with the homologues of $p34^{cdc2}$. For example, human cyclin E, which was produced in *E. coli,* bound to and activated the CDC2 H1 protein kinase in extracts from human G1 cells. In addition mRNAs of cyclin C and E are specifically present in the late G1 phase of the HeLa cell cycle and accumulate periodically through the cell cycle, peaking at different times in G1 phase (Koff et al., 1991; Lew et al., 1991). It is

interesting that the G1 cyclins in mice and humans, like those in budding yeast, have more than one form, for example, cyclin A, C, D, D1 and E in human cells. It is possible that the different forms of G1 cyclins are related to different stages in the G1 phase, to different cell lines or to affinities for different members of the cdc2/CDK family. They might also direct $p34^{cdc2}$ alternative substrates.

It is suspected that, although G1 activity of $p34^{cdc2}$ in complex with G1 cyclins can be measured with H1 histone as substrate, this is not the natural substrate. Phosphorylation of H1 histone is probably a natural activity at mitosis carried out by the mitotic cdc2/cyclin B complex and results in chromosome condensation. The substrates of cdc2 or CDK proteins at G1 phase are not yet known but probably include transcription factors for enzymes of DNA synthesis.

1.4 cdc2 protein kinase/cyclin-dependent kinase (CDK) family

1.4.1 Basic structural features of cdc2 protein kinase

As discussed in Chapter 1.2, *cdc2* is an essential cell cycle gene which controls the transition from G1 to S phase and the transition between late G2 and M phases in yeasts (Nurse and Bissett, 1981; Beach et al., 1982) and universally (Nurse, 1990). The protein encoded by *cdc2* gene ($p34^{cdc2}$) has certain conserved structural features (Lee and Nurse, 1987). These features are: (i) 34 kDa molecular size with sequence characteristics of a serine-threonine protein kinase; (ii) an ATP-binding segment that contains the tyrosine residue sequence GEGTYG; (iii) presence, at about 25 residues from the ATP-bind site on its C-terminal side, of a perfectly-conserved 16 amino acid sequence, EGVPSTAIRESLLKE (PSTAIR motif) which may be the cdc25 binding site; (iv) capacity to bind to $p13^{suc1}$ and to cyclin proteins. The activity of this protein kinase is temporally regulated and coincides with transitions between late G1 and S phase and between late G2 and M phases in fission yeast (Simanis and Nurse, 1986), in budding yeast (Wittenberg and Reed, 1988; Reed and Wittenberg, 1990) and in human cells (Draetta and Beach, 1988).

1.4.2 Universal cdc2 protein kinase

Not only is *cdc2* a key control gene of the yeast cell cycle but its homologues have also been found in higher eukaryotes (Nurse, 1990). A homologue of *cdc2* can be defined as a gene which has the ability to complement a defective *cdc2* and/or *CDC28* gene when introduced into yeast (Pines and Hunter, 1991b). In human cells, a homologue of the *cdc2* gene (cdc2 HS) from *H. sapiens* was cloned by expressing a human cDNA library in cells of *S. pombe* carrying a *cdc2* mutation (Lee and Nurse, 1987). The protein encoded by the human cdc2 gene has the same molecular weight as $p34^{cdc2}$, contains the PSTAIR motif and has a 63% identity of amino acid residues with the *S. pombe* enzyme (Lee and Nurse, 1987). The homologue of *cdc2* (*Dm* cdc2 (31E)) from *D. melanogaster* was cloned from a *Drosophila* cDNA library using probes obtained by PCR amplification. *Dm* cdc2 complements both a fission yeast *cdc2* mutation and a budding yeast *cdc28* mutation, the amino acid sequence of the Dm cdc2 contains the PSTAIR motif and in comparison with known cdc2 homologues it has at least 53% sequence identity (Lehner and O'Farrell, 1990). The chicken cdc2 DNA, which was cloned from a cDNA library, also encodes a PSTAIR-containing protein that rescues *cdc2* mutants in *S. pombe* (Krek and Nigg, 1989).

The enzyme activity of these homologues of $p34^{cdc2}$ is regulated in the cell cycle with a maximum of H1 histone kinase at mitosis (Arion et al., 1988; Brizuela et al., 1989; Draetta and Beach, 1988; Durkacz et al., 1986; Gautier et al., 1989; Labbe et al., 1988a, 1988b, 1989b; Langan et al., 1989; Lee et al., 1988; Norbury et al., 1991; Pines and Hunter 1990b; Roth et al., 1991; Rosenblatt et al., 1992)

1.4.3 Regulation of $p34^{cdc2}$ activity by phosphorylation

The regulation of $p34^{cdc2}$ protein kinase activity at mitosis by change in its phosphorylation is indicated by both genetic and biochemical evidence (Nurse, 1990).

Genetic analysis in the fission yeast *S. pombe* shows that the *cdc2* gene product is regulated by the *cdc25* gene (Fantes, 1979; Russell and Nurse, 1986) in opposition to the *wee1* and *mik1* genes (Russell and Nurse, 1987a). The product of the *cdc25* gene is a threonine-tyrosine phosphatase (Russell and Nurse, 1986; Gould et al., 1990; Dunphy

and Kumagai, 1991; Gautier et al., 1991; Kumagai and Dunphy, 1991). The products of the *wee1* and *mik1* genes are a protein kinases which phosphorylate both threonine and tyrosine residues (Russell and Nurse, 1987a; Featherstone and Russell, 1991).

A combination of biochemical and genetic evidence has shown that activation of $p34^{cdc2}$ in *S. pombe* at mitosis requires cdc25 activity and involves removal of phosphate from Tyr 15 (Gould and Nurse, 1989). In higher eukaryotes an additional phosphorylation site, which reinforces the effect of Tyr 15, is present at Thr 14. Animal cell mitosis is triggered by the removal of phosphate from both Tyr 15 and Thr 14 of $p34^{cdc2}$ that is complexed with cyclin B. The phosphorylation site at Thr 14, which is absent in *S. pombe,* may correlate with the presence of three cdc25 homologues in human cells. Subsequent to the analysis of the key roles of phosphorylation at Tyr 15 and Thr 14 it was established that phosphorylation of threonine at 167 in yeast (or Thr 161 in animal cells) is also essential. Chronologically in the cell cycle, phosphorylation of Thr 161/167 precedes that of Tyr 15 and Thr 14 and may be necessary to allow binding of cyclin B. The active form of $p34^{cdc2}$ at mitosis is phosphorylated at Thr 161/167 while phosphates at Tyr 15 and Thr 14 have been removed (Gould et al., 1991; reviewed by Norbury and Nurse, 1992; Fesquet et al., 1993). A discrete enzyme, named cdc kinase, has been purified from oocyte extracts and is responsible for phosphorylation of Thr 161/167, although it has not yet been identified by mutation (Solomon et al., 1992; Fesquet et al., 1993)). A phosphorylation that may be peculiar to birds has been observed to occur at serine 277 during G1 to S phases (Krek and Nigg, 1991a and 1991b). Evolution has conserved regulatory mechanisms, based on *cdc25* and *wee1/mik1* genes, in which tyrosine-threonine protein kinase, encoded by *wee1* and *mik1* genes, phosphorylates Tyr 15 and Thr 14 and threonine and tyrosine phosphatase activity, encoded by *cdc25* gene, then activates $p34^{cdc2}$ kinase by dephosphorylation of Tyr 15, Thr 14.

1.4.4 CDK enzymes

Recently, besides the homologues of $p34^{cdc2/CDC28}$, more protein kinases which are related in sequence to $p34^{cdc2}$ have been detected in eukaryotic cells (Pines and

Hunter, 1991b), and some of these protein kinases can bind G1 and/or mitotic cyclins, whereas others seem more distantly related because they do not form complexes with cyclins. The notion that cyclin-dependent kinases (CDKs) may be specialised for cell cycle catalysis in higher eukaryotes has arisen (Pines and Hunter, 1991b). The best characterised CDK is cdc2, which could be termed CDK1 although that name is rarely applied. The next most intensively studied CDK is CDK2 which is an animal protein kinase that is closely related in sequence to $p34^{cdc2}$, contains a PSTAIR motif and Tyr 15 and has the ability to bind to $p13^{suc1}$ and a preferential affinity for cyclin A (Pines and Hunter, 1991a). The CDK2XI (Eg1) gene was cloned from an egg cDNA library of *Xenopus*. The Eg1 protein kinase also contains PSTAIR region and Tyr 15 (Paris et al., 1991). CDK2XI that can bind to $p13^{suc1}$ beads and complexes with cyclin A has histone H1 protein kinase activity that oscillates in the cell cycle, being high in late S/early G2 phases and low in interphase (Gabrielli et al., 1992). A further possible CDK2 is the *Drosophila Dm* cdc2c (92f) gene from *D. melanogaster*, which was cloned from *Drosophila* cDNA library using probes obtained by PCR amplification. CDK2 Dm cdc2c has about 53% amino acid sequence identity with $p34^{cdc2}$ including the presence of the PSTAIR motif.

Failure to complement null mutations of *cdc2* or *CDC28* has been argued by Pines and Hunter (1991b) to be a diagnostic feature of CDK2 genes. The CDK2Hs gene from *H. sapiens* was reported to complement a *CDC28* mutation in *S. cerevisiae* but it could not complement the fission yeast *cdc2* mutation (Elledge and Spottswood, 1991; Meyerson et al., 1992). Further analysis showed that it could only complement one particular double mutation of *CDC28,* therefore its function appears to be distinct from *cdc2/CDC28* (Pines and Hunter, 1991a). The protein of 298 amino acids encoded by this gene shares 89% amino acid sequence identity with CDK2XI (Eg1) in *Xenopus* (Elledge and Spottswood, 1991; Meyerson et al., 1992). Human CDK2 activity oscillates during the cell cycle in cultured mammalian fibroblasts, increasing in late G1 or early S phase and declining during M phase, and active CDK2 is a complex of CDK2 with cyclin A (Rosenblatt et al., 1992). It is possible that CDK2 protein, which is active during S phase when cdc2 is not, is necessary for DNA synthesis. Human CDK2 can

also bind with cyclin E, and the complex of human CDK2 with cyclin E can perform START in budding yeast containing a *CDC28* mutation (Koff et al., 1991; Tsai et al., 1991).

More members of the CDK family have been discovered in higher eukaryotic cells. For example, about 10 human protein kinases have been detected amoung products of PCR reactions using cdc2 base primers and described as having some similarity in sequence to $p34^{cdc2}$ (Meyerson et al., 1992). Most of these protein kinases were previously unknown and their amino acid sequences share more than 50% identity with $p34^{cdc2}$. It was proposed that a large family of cdc2-related kinases in animal cells may be associated with the large family of cyclins that is now being recognised (Meyerson et al., 1992). Whether there are equivalents in plants and whether all or any are primarily involved in the cell cycle is unknown. In this thesis $p34^{cdc2}$-like protein has been analysed as a protein of close to 34 kDa with a PSTAIR sequence recognised by an antibody against the $p34^{cdc2}$ PSTAIR region, having the ability to bind to $p13^{suc1}$ and having H1 histone kinase activity that is calcium and cyclic AMP independent. The possibility cannot be eliminated that some of the protein and enzyme activity that was detected in plant extracts was contributed by variants of the cdc2 protein, like CDK2. However CDK proteins have not yet been identified in plants so there is no possibility of assessing their specific contribution, and, since their function in animal cells is a subset of the functions of $p34^{cdc2}$, the level of possible plant CDK proteins together with the level of plant $p34^{cdc2}$ protein is fully relevant to the occurrence of cell division activity.

1.5 Homologues of *cdc* proteins in plants

The unicellular plant *Chlamydomonas* is ideal for the study of cell cycle control since it is readily synchronised with high precision by darkness or absence of CO_2 and it does not form cell chumps, therefore individual cell size can be readily monitored (John, 1987). It has been observed that a control point in late G1 phase has a number of functional similarities with the START control of yeasts and other eukaryotes. These include (i) a transition into a DNA replication-cell division segment of the cell cycle in

which progress is independent of further net growth (ii) initiation of a doubling of DNA within 0.1 of a cell cycle and (iii) a dependency upon critical cell size which provides a means of coordinating the rate of cell division with the rate of growth (John, 1984, 1987). Recognition of the similarity of control points provided the first indication of plant cell cycle control mechanisms in common with other eukaryotes, which was confirmed at the molecular level by detection of $p34^{cdc2}$-like protein in *Chlamydomonas and higher plants* using antibody against PSTAIR-containing protein (John et al., 1989). At the molecular level it has been observed that the START control coincides with an increase in this PSTAIR-containing protein, which correlates with a similar increase in $p34^{cdc2}$ in mammalian cells (Lee and Nurse, 1987). Furthermore an extensive phosphorylation of the $p34^{cdc2}$-like protein was restricted to the DNA replication-nuclear division segment of the cell cycle (John et al., 1989) as in human cells (Draetta and Beach, 1988).

An obligate coupling of cyclin degradation with $p34^{cdc2}$ catalytic inactivation for transition from metaphase to anaphase has been detected in *met1*, a metaphase arresting conditional *cdc* mutation of *Chlamydomonas* (John and Wu, 1992). Taken together with the recent evidence for cyclical changes in cyclin B levels in synchronously dividing alfalfa (Hirt et al., 1992) and the presence of plant $p13^{suc1}$-like proteins (John et al., 1991) it seems likely that the main features of $p34^{cdc2}$ activity control may be similar in higher plant cells and in other eukaryotes.

In higher plants homologues of the *cdc2* gene have recently been found, by polymerase chain reaction or by degenerate oligonucleotide probing, in cDNA libraries of pea, *Arabidopsis*, alfalfa, and maize (Colasanti et al., 1991; Feiler and Jacobs, 1991; Ferreira et al., 1991; Hirt et al., 1991). The homologues of maize and alfalfa cdc2 rescue *S. cerevisiae CDC28-1N* and *S. pombe cdc2-33* mutants, respectively (Colasanti et al., 1991; Hirt et al., 1991). The homologue of the cdc2 gene from *Arabidopsis* partially complements the *CDC28* mutation in *S. cerevisiae* (Ferreira et al., 1991), and the CDC2a cDNA from *Arabidopsis thaliana* when expressed in *S. pombe* corrects the elongated yeast cell morphology, which is caused by the temperature-sensitive *cdc2-33* mutation (Hirayama et al., 1991). All detected higher plant homologues of *cdc2* encode

proteins carrying the complete "PSTAIR" peptide sequence motif (EGVPSTAIREISLLKE) which is unique to $p34^{cdc2}$ and to CDK2 protein kinase (Pines and Hunter, 1991b). PSTAIR peptide was used to raise the PSTAIR antibody used in this thesis and at 25 nM peptide competed for binding to the plant proteins recognised in this thesis as PSTAIR-containing. The amino acid sequence of the $p34^{cdc2}$ homologue from *Zea mays* is 64% identical to human $p34^{cdc2}$ and 63% identical to $p34^{cdc2}$ protein in yeast (Colasanti et al., 1991). In addition, all of the near full- or full-length clones encode the ATP-binding "GEGTYG" motif which contains the tyrosine 15 residue that can be regulated by phosphorylation, although protein tyrosine phosphorylation has yet to be demonstrated directly in higher plants (Colasanti et al., 1991: Feiler and Jacobs, 1991; Ferreira et al., 1991; Hirayama et al., 1991; Hirt et al., 1991). Indirect evidence of phosphorylated isoforms of plant $p34^{cdc2}$ has been obtained by immunoblot assays that reveal differences in electrophoretic mobility consistent with phosphorylation (Feiler and Jacobs, 1990; Gorst et al., 1991; Hirt et al., 1991). The kinase activities of $p34^{cdc2}$ homologues have been detected, by purification of plant homologues of $p34^{cdc2}$ with $p13^{suc1}$-Sepharose and using histone H1 as substrate, in meristematic tissue of wheat, maize, *Arabidopsis* and pea (John et al., 1991; Feiler and Jacobs, 1991; Colasanti et al., 1991; Ferreira et al., 1991). These results have further indicated the possibility that $p34^{cdc2}$ is involved in the control of cell division in higher plants.

Likely homologues of the *suc1* gene product, $p13^{suc1}$, were detected in wheat, pea and *Chlamydomonas* using affinity-purified antibody raised against the fission yeast $p13^{suc1}$ protein, and it was suggested that the distribution of $p13^{suc1}$ homologues in plants is likely to be ubiquitous (John et al., 1991). The evidence that the $p34^{cdc2}$-like protein from wheat, pea and the green alga *Chlamydomonas* can bind to $p13^{suc1}$ protein encoded by the fission yeast *suc1* gene indicates that an association between plant homologues of $p34^{cdc2}$ and $p13^{suc1}$ is probably involved in plant mitosis. It is likely that, as in fission yeast (Hayles et al., 1986b; Moreno et al., 1989), $p13^{suc1}$ may be essential for the inactivation of $p34^{cdc2}$ during mitosis, and for the completion of the later stages of mitosis and the reestablishment of the interphase cytoskeleton.

Genetic information for cyclin-like proteins has been detected in plants but the species of cyclin with which specific plant genes may be homologous has been difficult to determine with certainty, especially in the case of possible G1 cyclins that are difficult to characterise in animal cells solely on the basis of their sequence. Homologues of probable mitotic cyclins were detected in carrot, soybean, alfalfa and pea by polymerase chain reaction and by degenerate oligonucleotide probing (Hata et al., 1991; Hirt et al., 1992). The carrot cyclin is similar to A-type cyclin, and the soybean cyclin is similar to B-type cyclins although they have divergent amino acid sequences in the portion that is most highly conserved in known cyclins (Hata et al., 1991). In cyclin cycMS1 and cycMS2 genes from alfalfa, the highest similarity to type B cyclins is found in the cycMS2 gene, and the predicted amino acid sequence of the cycMS1 gene shows 25 to 35% similarity to all types of cyclins (Hirt et al., 1992). Both cycMS1 and cycMS2 genes in alfalfa are expressed in dividing suspension culture cells but cease to be expressed when the cells enter stationary phase and in synchronised cells the maximal abundance of the mRNAs of cycMS1 and cycMS2 between late G2 and M phases is consistent with a cyclin B function (Hirt et al., 1992). Microinjection of mRNA for the soybean cyclin induces the maturation of *Xenopus* oocytes (Hata et al., 1991). It is therefore possible that plant cyclins are associated with plant homologues of $p34^{cdc2}$ protein during the cell division cycle in plants and direct evidence that is consistent with this has come from the detection of a 56 kDa protein that copurifies with wheat $p34^{cdc2}$, through DEAE cellulose and $p13^{suc1}$-affinity chromatography, and is recognised by antibody against the cyclin B of fission yeast, $p56^{cdc13}$ (John et al., 1993a). These results therefore support the possibility that the main characteristics of $p34^{cdc2}$ activity control in plants may be similar to those in yeasts and animal cells.

Homologues of $p34^{cdc2}$ protein kinase, and probably of mitotic cyclin and of $p13^{suc1}$ have been discovered in plants, but to date no evidence has been published that shows how they may participate in the control of cytoskeletal elements, such as the preprophase band and phragmoplast that are unique to plants and transiently formed in the cell cycle, nor how they participate in the switch between proliferation and

differentiation in organogensis. Some evidence relevant to these questions is advanced in this thesis.

1.6 Cell division and differentiation in higher plants

The developmental strategies of higher plants bear little resemblance to those of unicellular eukaryotes or vertebrate animals. Higher plants are "perpetually embryonic" in that they produce new organs throughout their lives, however the organs are often of a determined size therefore cell division can also be turned off. Cell division in higher plants is more discretely localised to meristem regions that supply cells for both the formation of new organs throughout the organism's life and for radial expansion of its axial components. Cells that are displaced from the meristems cease division and are able to develop specialised structures and functions.

1.6.1 Meristems, cell division and cell differentiation

Cell division in plant meristems has some characteristics that are different from those in unicellular eukaryotes or vertebrate animals. For example, cell division in root meristems is often asymmetric and cells forming different tissues may divide at different sizes (Davidson, 1991). Another peculiar feature of root meristems is the quiescent centre, a group of cells predominantly in G1 phase which are either cycling slowly or are non-cycling in G0 phase, however the quiescent centre can regenerate whole plants when dissected and cultured *in vitro* (Feldman, 1976). Shoot meristems can show determinate growth when giving rise to vegetative leaves and floral parts. In leaf meristems division activity is of a relatively short duration and is followed by a phase of mesophyll cell expansion. In monocotyledonous leaves there are localised basal meristems, whereas in dicotyledonous leaves, there may be several separate clusters of dividing cells. Although the H1 histone activity of $p34^{cdc2}$ homologues has been detected in meristematic tissue of wheat, pea, maize and *Arabidopsis* (John et al., 1991; Feiler and Jacobs, 1991; Colasanti et al., 1991; Ferreira et al., 1991) there is no published evidence concerning whether changes in activity of $p34^{cdc2}$ underly changes in

meristem activity. This thesis will provide evidence that changes in $p34^{cdc2}$ level and activity may determine division activity.

1.6.2 Changes in the cytoskeleton during the plant cell cycle

Significant changes to the cytoskeleton occur during the plant cell cycle. Microtubules are closely associated with the outer cell membrane during interphase and in G2 phase these become increasingly concentrated into a preprophase band (PPB) that is formed in the cortical cytoplasm and encircles the nucleus (Pickett-Heaps and Northcote, 1966; Gunning et al., 1978). The PPB is unique to plant cells and its location at early prephase marks the site at which the new cross wall will eventually fuse with the old. PPBs are cleared from this site at metaphase, when formation of the new wall is imminent (Baskin and Cande, 1990; Mineyuki and Gunning, 1990). At telophase, cytokinesis begins with the appearence of phragmoplast microtubules between the daughter nuclei, where the cell plate is forming (Hepler and Newcomb, 1967; Hepler and Jackson, 1968). In late telophase, as the cell plate is growing, the phragmoplast will, if necessary, undergo extensive re-orientation to contact the side walls at the site predicted by the PPB (Palevitz and Hepler, 1974; Gunning, 1982). The molecular signals, which control the formation and disassembly of PPB and phragmoplast microtubules, are still unknown but this thesis will provide evidence that is consistent with possible involvement of $p34^{cdc2}$ in PPB disassembly.

1.6.3 Principal control points in cell division cycle

The principal control point hypothesis has been put forward by Van't Hof (Van't Hof, 1974) and states that cell division in plant tissues is regulated by factors that operate during G1 and G2 phases. Under conditions where cell division ceases these factors become limiting, causing cell cycle arrest in G1 or G2 phase. Although the hypothesis was based on observations made on cells of complex tissues such as pea root meristems, a similar tendency to arrest in G1 or G2 rather than S or M phase was found in some plant cell suspension cultures. For example, *Acer pseudoplatanus* cell suspension culture suffering phosphorus or carbohydrate starvation arrests in G1 and G2

phases in an approximate ratio of 4 to 1, whereas nitrogen starved cells accumulate virtually exclusively in G1 (Gould et al., 1981). So it is evident that control points in cell division cycle of higher plants are located in G1 and G2 phases but the number of control points in each phase and their location relative to be beginning and end of these phases was not known. The similarity of control points and of cell cycle proteins in the unicellular plant and in yeasts lead John et al (1989) to propose that the principle control point hypothesis should be refined to recognise the G1 control as probably equivalent to START in yeasts and the G2 control as mitotic initiation. This view is supported in the present thesis by the preferential arrest of tobacco cells in late G2 just prior to $p34^{cdc2}$ activation.

1.6.4 Cell division, cell differentiation and phytohormones

The relationship between higher plant cell division and the presence of added phytohormones was investigated in this thesis for three reasons. First, hormone stimulation provides a means to induce cell division activity and, in this circumstance, the investigation of whether key cell division proteins are induced and become enzymically active provides a test of whether they might act as control elements in plants. Second, the investigation of single hormones and hormone mixtures for their effects on synchronous cultures provides an indication of whether particular cell cycle events are specifically dependent on particular types of hormone. Third, information gathered concerning the effects of hormones that have been experimentally supplied to cells and tissues indicates possible mechanisms of division control in intact plants. Rigorous evaluation of which possible controls may actually be employed in intact plants and under what developmental circumstances, such as in normal development or wound response, is a problem outside the scope of this thesis. Its investigation would require specialised techniques for measurements of *in vivo* concentration of free hormones that could not be undertaken in addition to the investigations present here. Because of its technical difficulty this remains a major unsolved problem in plant biology.

Although much remains to be resolved concerning the possible similarities between the model plant systems studied here and phytohormone actions in the intact plant, it is

hoped that this thesis can present interesting new data concerning the involvement of key cell division proteins that were initially identified by genetic analysis in yeast cells.

1.7. Aim of this thesis

The foregoing survey of cell cycle mechanisms and of cell cycle control in differentiation in higher plants gives rise to many questions. Those that were investigated in this thesis are: (i) when is $p34^{cdc2}$ protein catalytically active in the higher plant cell cycle; does catalytic activity correlate with the the initiation of cell cycle events, especially, at mitosis? (ii) what controls the changes of the cytoskeleton, especially of preprophase band of microtubules in the cell division cycle? (iii) what controls cell differentiation and dedifferentiation? (iv) what are the functions of phytohormones in cell division, cell differentiation and dedifferentiation?

Most studies to date have focused on establishing a one-to-one correspondence between characterised cell cycle regulators in the model yeasts and their putative counterparts in the plant kingdom so that the questions described above still have to be answered. In this thesis, I have used the cell suspension culture of *Nicotiana plumbaginifolia* as a model system to study the level and enzyme activity of $p34^{cdc2}$-like protein in synchronous cultures that were obtained by the use of the DNA polymerase inhibitor Aphidicolin or by nutrient limitation (Chapter 3), to study the relationship between $p34^{cdc2}$-like protein kinase activity, protein phosphorylation and the mitotic changes in chromatin structure and in PPB microtubule disassembly (Chapter 4). Suspension cultures were also used to study the possible relationship between levels of $p34^{cdc2}$-like protein and the regenerability of short-term and long-term cell suspension cultures (Chapter 5). For studying the mechanisms by which phytohormones may influence dedifferentiation of cells in tissue and the relationship between the $p34^{cdc2}$-like protein kinase activity and auxin and cytokinin presence, tobacco stem pith was used (Chapter 6). Suspension cultures of *N. plumbaginifolia* were used to invesigate which cell cycle events might be specifically dependent upon stimulation by particular hormones (Chapter 6).

CHAPTER 2

MATERIALS AND GENERAL METHODS

2.1. Materials

2.1.1. Chemicals

Unless otherwise stated all biochemicals were supplied by the Sigma Chemical Company Ltd. Analytical grade general laboratory chemicals were supplied by Ajax Chemicals, Clyde Industries Ltd. Auburn Australia.

2.1.2. Organisms

2.1.2.1. Cell suspension culture of Tobacco (*Nicotiana plumbaginifolia*)

A long term cell suspension culture (NpT5) of *N. plumbaginifolia* was obtained from Dr Phillip J. Larkin, CSIRO Division of Plant Industry, Canberra, Australia. An equivalent short term suspension culture was prepared from leaf tissue derived from the same stock of seed held at CSIRO plant industry.

2.1.2.2. Tobacco Wisconsin 38

Tobacco (*Nicotiana. tabacum*) cv Wisconsin Havana 38 was obtained from Dr D. S. Letham, Plant Cell Biology Group, Research School of Biological Sciences ANU, Canberra, Australia.

2.1.2.3. Bacteria

Bacterial strain BL21 (DE3), obtained from Dr J. Hayles, Oxford University, U. K., was used in the overexpression of $p13^{suc1}$.

2.1.3. Culture media

2.1.3.1. CSV medium (Lorz et al., 1983)

CSV medium contained 88 mM sucrose, 15.5 mM NH_4NO_3, 25mM KNO_3, 4 mM $NH_4H_2PO_4$, 0.14 mM $CaCl_2.2H_2O$ and and 1.6 mM $MgSO_4.7H_2O$ together with Micro elements and Organic nutrients. It was prepared by mixing in 1 l final volume 50 ml of 20 x Macro elements, 1 ml of 1000 x Micro elements, 1 ml of 1000 x Na_2EDTA, 1 ml of

$FeSO_4.7H_2O$, 1 ml of 1000 x Organic nutrients solution, 30 g sucrose, 1 g myo-inositol, 200 µl of 45.24 mM 2, 4-D, 46.4 µl of 5 mM kinetin and adjusted to pH 5.8-6.0.

20 x Macro elements consisted of 24.8 g NH_4NO_3, 50.1 g KNO_3, 9.2 g $NH_4H_2PO_4$, 4.0 g $CaCl_2.2H_2O$, 8.0 g $MgSO_4.7H_2O$ and final 1 l of volume with H_2O. 1000 x Micro elements consisted of 1.3 g $MgSO_4.7H_2O$, 0.5 g H_3BO_3, 0.1 g $ZnSO_4.7H_2O$, 0.1 g KI, 20 mg $CuSO_4.5H_2O$, 10 mg $Na_2MoO_4.2H_2O$ and 10 mg $CaCl_2.6H_2O$ and final 100 ml of volume with H_2O. 1000 x Na_2EDTA solution is 2 g Na_2EDTA in 100 ml of H_2O. $FeSO_4.7H_2O$ solution is 0.75 g $FeSO_4.7H_2O$ in 50 ml of H_2O. 1000 x Organic nutrients solution consisted of 1.0 g thiamine-HCl, 1.0 g nicotinic acid, 0.1 g pyridoxine-HCl in a final volume of 100 ml.

2.1.3.2. MS medium

MS medium contained 88 mM sucrose, 20 mM NH_4NO_3, 19 mM KNO_3, 0.3 mM $CaCl_2.2H_2O$, 1.5 mM $MgSO_4.7H_2O$ and 0.13 mM KH_2PO_4. It was prepared by mixing in a final one litre; 50 ml of 20 x Macro elements, 1 ml of 1000 x Micro elements, 1 ml of 100 x Organic nutrients solution, 0.5 ml of 200 x Fe-EDTA solution, 1 g myo-inositol, 30 g sucrose, and was adjusted to pH 5.9-6.0. For solid MS medium 8 g Bacto-Agar was added per litre MS.

20 x Macro elements consisted of 33 g NH_4NO_3, 8.8 g $CaCl_2.2H_2O$, 38 g KNO_3, 7.4 g $MgSO_4.7H_2O$, 3.4 g KH_2PO_4 and in a final volume of 1 l. 1000 x Micro elements consisted of 0.622 g H_3BO_3, 2.23 g $MnSO_4.4H_2O$, 0.86 g $ZnSO_4.7H_2O$, 83 mg KI, 25 mg $Na_2MoO_4.2H_2O$, 2.5 mg $CuSO_4.5H_2O$, 2.5 mg $CoCl_2$ in a final volume of 100 ml. 100 x Organic solution consisted of 5 mg nictonic acid, 5 mg pyridoxine-HCl, 1 mg thiamine-HCl, 20 mg glycine in a final volume of 100 ml. 200 x Fe-EDTA solution contained 0.671 g Na_2EDTA, $FeCl_3.6H_2O$ in 100 ml of H_2O.

2.1.3.3. LB medium (Luria-Bertani medium)

1 l of LB medium contained 10 g Bacto-tryptone, 5 g Bacto-yeast extract, 10 g NaCl and was adjusted to pH 7.0 with 5 N NaOH.

2.2 Methods

2.2.1. Cell suspension cultures of tobacco (*N. plumbaginifolia*)

Cell suspension cultures of *N. plumbaginifolia* were maintained by transfer of 8 ml of culture to 40 ml fresh CSV medium every 4 days. Cultures were incubated at 25°C and 100 rpm on a rotary shaker.

2.2.2. Quantification of nuclear DNA

2.2.2.1. Staining of nuclear DNA with propidium iodide for quantification

Cells were fixed in 25% ethanol for 5 min, dehydrated in 50% ethanol for 2 min then in 80% ethanol for 2 min and pelleted by spinning down at 4000 rpm 2 min in a Microfuge (Beckman) and resuspended in 80% ethanol. Cells could be stored at -20°C for a few days at this stage. Before staining, cells were rehydrated in 50% and 25% ethanol then spun down and resuspended in 5 mM tris-HCl, 1 mM EDTA pH 7.4. RNA in cells was removed by adding a final concentration of 1 mg/ml of RNAase (bovine pancreas, 5 x crystallised type 1A, Sigma), which was heated in 10 mM tris-HCl pH 7.4 solution at 100°C for 15 min before use to cell suspension and incubating at 37°C for 30 min. Cells were rinsed in 10 mM tris-HCl pH 7.4 and then dried briefly onto cover slides that had previously been prepared by spreading with chicken erythrocytes that were allowed to dry. DNA was stained overnight with 2.5 µg/ml propidium iodide in 5 mM tris-HCl 1 mM EDTA (pH 7.4) 50% ethanol. Excess propidium iodide solution was removed and cells were mounted in a minimum volume of glycerol under a cover slide sealed with nail varnish.

2.2.2.2. Quantification of fluorescence from nuclear DNA

A Nikon Optiphot microscope was used with a rhodamine filter set giving excitation in the range 510-560 nm with a 580 nm dichroic mirror and 590 nm barrier filter. Images obtained from an SIT television camera (Dage MTI, Michigan City, IN, U.S.A.) were digitised and recorded using an Image-1 image processing attachment and analysed using software Image-1 Version 4.0 (Universal Imaging Corporation, 502

Brandywine Pkwy West Chester, PA 19380, U.S.A.). A measurement of nuclear brightness was made in a minimum area enclosing each whole nucleus, and from each cell an estimate of background intensity was obtained by measuring three areas of the same size in the immediately adjacent cytoplasm at approximately 120° spacing around the nucleus. Average background intensity was usually less than one sixth of nuclear intensity and was subtracted. Only nuclei in focus were measured and the microscope was refocussed until all nuclei in each field of view had been measured.

2.2.3. Staining of chromatin using DAPI

Cells were pelleted by centrifugation at 4000 rpm in a Microfuge and fixed by suspending in 4% (w/v) paraformaldehyde in PBS for 1h at room temperature. After rinsing for 3 x 10 min in PBS, which consists of 1.5 mM KH_2PO_4, 8.0 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl and pH 7.4, cells were stained by suspending in 0.2 µg / ml 4', 6' diamidino-2-phenylindole (DAPI) for a few min then placed on slides that had previously been coated with poly-L-lysine (M_r 540,000). After excess liquid was removed, cells were mounted in Citifluor antifade mounting medium (AFI; City University, London, U.K.) and cover slips were sealed with nail varnish. Observations were made with a Nikon Optiphot microscope using a standard UV filter set. Pictures were taken on Kodak Tmax 400 film rated at 400 ASA.

2.2.4. Counting of cell number in cell suspension culture with haemocytometer

Nuclei were stained with DAPI then cells were resuspended in PBS to a volume equal to that in which they were sampled. Cell number was determined by placing 10 µl of cells resuspended in PBS after staining with DAPI on a haemocytometer and counting all nuclei with a Nikon Optiphot microscope using a standard UV filter set. The sample was diluted with PBS if there were far more than 500 cells in 10 µl of sample. Cell number density per ml was calculated by cell number in 10 µl x 100.

2.2.5. Enzyme extraction from plant cells

2.2.5.1. Protein extraction from cell suspension culture of *N. plumbaginifolia*

Cells were pelleted by centrifugation at 4000 rpm for 2 min in a Microfuge and rinsed in 10 mM tris-HCl pH 7.4 twice and then frozen in liquid nitrogen. Cells were then ground in liquid nitrogen using pestle and mortar. Protein was extracted from the resulting powder by vigorously mixing 0.1g of cell powder with 100 µl of RIPA buffer pH 7.4 for 3 x 20 sec at 0°C. RIPA buffer consisted of 20 mM tris-HCl, 5 mM EDTA, 100 mM NaCl, 0.1 % Tween 20, 1 mM dithiothrietol (DTT), 10 µM leupeptin, 10 µM pepstatin, 10 µM NaF, 1 mM EGTA, 1 mM sodium pyrophosphate, 1 mM β-glycerophosphate, to which was added immediately before use 1 mM sodium orthovanadate and 200 µM phenylmethysulfonyl fluoride (PMSF). The protein extract was centrifuged at 4000 rpm for 5 min in Microfuge. For electrophoresis 100 µl of protein supernatant was mixed with 100 µl Sx2 SDS Laemli sample buffer, containing 125 mM tris-HCl pH6.8, 4 % (w/v) SDS, 20 % (v/v) glycerol, 2 % (v/v) mercaptoethanol, 0.002 % bromophenol blue and boiled for 3 min.

Protein amounts in the protein extract was measured by Coomassie Brilliant blue assay (2.2.6.1) or Ponceau staining assay (2.2.6.2).

2.2.5.2. Protein extraction from pith tissue or pith-derived callus of tobacco Wisconsin 38

Pith tissue or callus was frozen and ground in liquid nitrogen. Protein was extracted by vigorous mixing of 0.6 g pith tissue powder or pith callus powder with 4 ml MES buffer pH 6.0 containing 25 mM 2 (N-morpholino) ethanesulfonic acid (MES), 5 mM EDTA, 100 mM NaCl, 0.1 % Tween 20, 1 mM dithiothrietol, 10 µM leupeptin, 10 µM pepstain, 10 µM NaF, 1 mM EGTA, 1 mM sodium pyrophosphate, 1 mM β-glycerophosphate, to which was added immediately before use 1 mM sodium orthovanadate and 200 µM PMSF. The extract was centrifuged at 10,000 rpm in a JA 20 rotor for 10 min, and to about 4 ml of supernatant was added 20 ml -20°C acetone to precipitate the protein. Protein was collected by centrifugation at 10,000 rpm in a JA 20 rotor for 10 min and the pellet was redissolved in 0.3 ml MES buffer. The protein

concentration was measured by Coomassie Brilliant blue assay (2.2.6.1) or Ponceau staining assay (2.2.6.2), the latter being adopted as more acurate for extracted pith protein.

2.2.6. Quantification of protein in extracts

2.2.6.1. Quantitative assay of protein with Coomassie Brilliant blue

The assay was based on complexing protein with Coomassie brilliant blue G and the extinction coefficient of the dye-protein complex solution remained constant over the protein concentration range of 0.8 to 10 µg/ml of solution (Spector, 1978).

1. Reagents for protein assay

Dye reagent was made by dissolving 0.05g Coomassie Brilliant blue G250 (Bio-Rad 161-0406) in 25 ml 95% ethanol with thorough mixing, then adding 50 ml of 85% phosphoric acid and making to 500 ml with H_20.

2. Standard curve

Ovalbumin at 100 µg/ml in H_20 was used to make calibration assays containing 0, 5, 15, 50, 75, 100 µl of 100 µg/ml ovalbumin, 100 µl RIPA buffer and each was supplement to a final volume of 200 µl with H_20. Each 200 µl of mixture received 1 ml dye reagent and was kept at room temperature for 5 min before measurement of OD at 595 nm. A standard curve was prepared with each set of assays.

3. Unknown samples

For each unknown, assays containing 5, 25 and 100 µl of extract in RIPA buffer and 100 µl of H_20 were supplemented to a final volume of 200 µl with RIPA buffer. Each assay of standard and unknown therefore contained a total of 100 µl of RIPA buffer. Each mixture received 1 ml of dye reagent and was kept at room temperature for 5 min prior to measurement at 595 nm.

2.2.6.2. Assay by electrophoresis transferr and Ponceau S stain

Extracts from pith tissue contained fragments, probably of wall material, that reacted to some extent with the Coomassie dye reagent and therefore prevent accurate

protein measurement. Accurate estimates were obtained by performing electrophoresis and protein transfer to nitrocellulose as in Western blotting then staining with Ponceau S.

1. Standard protein solution

To provide a calibration for the efficiency of protein transfer and of Ponceau S binding a standard protein mixture, containing the range of molecular weights that is most abundant in natural plant extracts, was always electrophoresed in parallel with unknown samples on every gel. In the standard protein mixture a total concentration of 2400 µg protein per ml was made by pooling 5 mg each of nine proteins, which were human transferrin (Sigma, T-4515), β-glucosidase (BDH 0329280), phosphitin (Sigma, egg vitellin, P-1253), bovine milk β-lactoglobulin (Sigma, L-7880), horse pancreas α-amylase (Sigma, A-6255), egg ovalbumin (Sigma, A-5503), bovine serum albumin (Sigma, A-7638), trypsin inhibitor (Sigma, T-9003) and horse heart cytochrome-C (Sigma, C-2506) with 9.1 ml of H_20 and 9.3 ml of Sx2 SDS sample buffer and heating in a boiling water for 5 min.

2. Electrophoresis of proteins in 12% SDS-acrylamide gel

Wells were loaded with 10, 20, 30 and 60 µl of standard protein solution and with 25, 50, 75 and 100 µl of plant extract of unknown protein concentration, both mixed with SDS sample buffer on a 12% SDS-acrylamide gel. The method of running is given in section 2.2.7. Proteins were transferred to 0.45 µm nitrocellulose as described in section 2.2 8.

3. Ponceau S staining, nitrocelulose labelling and photographing

The nitrocellulose was stained for 30 min with 0.4% Ponceau S in 3% TCA. Excess stain was washed off by placing the nitrocellulose in 500 ml of 0.1% acetic acid and shaking for 10 min. The washing was repeated till the background of nitrocellulose was white. Nitrocellulose was transferred to a glass plate, labelled with gel number and identity of samples in individual tracks and kept wet with 0.1% acetic acid until photographed in Tech - Pan Kodak film.

4. Quantification of bound Ponceau S stain

Tracks were cut out, including a background strip carrying no protein. Each strip was put in separate clean 1 dram vial containing 2 ml of 50 µM tris-HCl pH 9.3 and was

shaken for 10 min. The amount of Ponceau S stain that had been bound to protein was determined by reading OD at 518 nm.

5. Standard curve

A standard curve was obtained by plotting measured the absorptance at 518 nm from strips of N/C on which 0, 36, 52, 72, 144 µg of proteins run. From this line, the amount of protein present in samples of extract could be deduced.

2.2.7. SDS-acrylamide gel electrophoresis

1. 12% acrylamide gel contained 9 ml of 40% acrylamide 1.07% bis-acrylamide, 5.55 ml of H_20, 15 ml of 0.75M tris-HCl pH 8.8, 300 µl of 10% sodium dodecyl sulfate (SDS), 15 µl of N, N, N', N'-tetramethylethylenediamine (TEMED) and 113 µl of 15% (w/v) ammonium persulphate.

2. 15% acrylamide gel contained 11.25 ml of 40% acrylamide 1.07% bis-acrylamide, 3.3 ml of H_20, 15 ml of 0.75M tris-HCl pH 8.8, 300 µl of 10% SDS, 15 µl of TEMED, 113 µl of 15% (w/v) ammonium persulphate.

3. 10-15% linear gradient acrylamide separating gel was made by mixing 10% acrylamide monomer solution and 15% acrylamide solution using a gradient mixer block and variable speed laboratory motor (Tri-R Stir-R model S63C) and an LKB pump with 4/25 gearbox. 10% acrylamide monomer solution contained 3.75 ml of 40% acrylamide 1.07% bis-acrylamide, 3.7 ml of H_20, 7.5 ml of 0.75 M tris-HCl pH 8.8, 200 µl of 10% SDS, 7.5 µl of TEMED, 14 µl of 15% (w/v) ammonium persulphate; 15% of acrylamide monomer solution contained 5.6 ml of 40% acrylamide 1.07% bis-acrylamide, 1.7 ml of H_20, 7.5 ml of 0.75M tris-HCl pH 8.8, 200 µl of 10% SDS, 7.5 µl of TEMED, 14 µl of 15% (w/v) ammonium persulphate.

4% stacking gel contained 1.5 ml of 40% acrylamide 1.07% bis-acrylamide, 11.4 ml of H_20, 1.8 ml of 1 M tris-HCl pH 6.8, 150 µl of 10% SDS, 15 µl of TEMED, 37.5 µ l of 15% (w/v) ammonium persulphate.

4. Sample was mixed with an equal volume of SDS sample buffer (Sx2), which contained 0.125 M tris-HCl (pH 6.8), 4% (w/v) SDS, 20% (v/v) glyerol, 2% (v/v) mercaptoethanol and 0.002% bromophenol blue.

Electrophoresis buffer consisted of 25 mM tris-HCl pH 8.3, 193 mM glycine and 0.1% SDS. Current was held at 25 mA per gel during stacking and increased to 35 mA per gel when the dye front reached the separating gel. Electrophoresis was stopped when the dye was within 5-10 mm of the bottom of the gel.

2.2.8. Western blotting and immuno-detection in blots

The conventional methods of Western blotting were adopted (Towbin et al., 1979. For brevity in the description of the method buffers are described by abbreviations and these are therefore explained first.

1. Buffers

(1). Transfer buffer (TB); contained 25 mM tris-HCl, 192 mM glycine, 20% methanol and pH 8.3.

(2). Antibody washing buffer (TBS 20); contained 10 mM tris-HCl pH 7.4, 0.15 M NaCl and 0.05% Tween20.

(3). Blocking solution; contained 10 mM tris-HCl pH 7.4, 0.15 M NaCl and 5% skim milk powder.

(4). Antibody dilution buffer (TBSBT); contained 10 mM tris-HCl pH 7.4, 0.15 M NaCl, 0.05% Tween 20 1% bovine serum albumin (BSA) 0.05% sodium azide.

(5). Alkaline phosphatase substrate buffer; contained 0.1 M tris-HCl pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2.6\ H_20$.

(6). Alkaline phosphatase substrate was prepared by adding 5 ml of alkaline phosphatase substrate buffer to 22 µl of nitroblue tetrazolium chloride (NBT) solution, which is 100 mg in 1.3 ml 70% dimethylformide, and 16 µl of 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (BCIP) solution, which is 100 mg in 2.0 ml 100% dimethylformamide.

2. Blotting, staining and photographing

Proteins were transferred for 6h at 180 mA onto 0.45 micron nitrocellulose, the nitrocellulose was immersed in Ponceau S solution (2.2.6.2) for 15-30 min and unbound stain was washed off with H_20 twice until the background of nitrocellulose was white then photographed using Tech-Pan Kodak film with a green filter.

3. Blocking. Whole nitrocellulose (or nitrocellulose strips) was put in blocking solution for at least 1h with shaking then rinsed 3 x 5 min in antibody washing buffer with shaking.

4. Immuno-detection in blots

(1). After blocking, nitrocellulose was arranged on Nescofilm and overlaid at room temperature for 1-2h with anti-PSTAIR antibody solution diluted with TBSBT to 1:500 relative the concentration in the original serum. The unbound first antibody was rinsed off by 3 x 10 min shaking in antibody washing buffer, which for the first two washes contained 0.05% Tween20 in TBS and for the last wash contained 0.2% Tween20 in TBS.

(2). Reaction with second antibody

(a). Anti-rabbit IgG with coupled alkaline phosphatase

After reaction with the first antibody the nitrocellulose was incubated in second antibody solution; anti-rabbit IgG (F(Ab) fragment) produced in goat, conjugated with alkaline phosphatase (Sigma A-8025) diluted to 1/4000 in TBSBT, at room temperature for 1 hour. The nitrocellulose was rinsed 2 x 10 min with 0.2% Tween20 in TBS, once with no Tween-TBS for 10 min and then with alkaline phosphatase substrate buffer for 5 min until reaction at positive bands was clear. The nitrocellulose was immediately removed into H_20 to stop the reaction.

(b). ^{125}I anti-rabbit-IgG antibody

Alternatively, after reaction with anti-PSTAIR first antibody, the nitrocellulose was washed with TBS 20 and put in a plastic box with ^{125}I anti-rabbit IgG antibody produced in mouse (Amersham, IM1340) diluted to 0.5 μci/ml with TBSBT for 2h. The unbound second antibody was washed off by 3 x 10 min with 0.2% Tween20 in TBS with shaking. The nitrocellulose was dried and an image of the radioactivity was obtained by exposure in a phosphorImager.

2.2.9. Affinity purification of anti-EGVPSTAIREISLLKE antibody (PSTAIR antibody)

1. Preparation of purified $p34^{cdc2}$ on nitrocellulose for antibody binding

300 µl of "semipure $p34^{cdc2}$", which was the product of the yeast *cdc2* gene over-expressed in *E. coli* (about 100 µg of $p34^{cdc2}$), kindly provided by F. J. Sek, Plant Cell Biology Group, R.S.B.S was loaded on two 12% SDS-acrylamide gels after mixing with equal volume of Sx2 SDS sample buffer. The 34 kDa cdc2 protein band was cut out as 1 cm wide strip after transfer to nitrocellulose and staining with Ponceau S. The strips of nitrocellulose (N/C) which contained purified $p34^{cdc2}$ were blocked in blocking solution (2.2.8) washed in H_20 dried and later used to purify PSTAIR antibody.

2. Binding PSTAIR antibody to purified $p34^{cdc2}$ nitrocellulose. 20 µl of PSTAIR antibody was diluted into 10.4 ml with TBS 1% BSA 0.05% Tween20 0.02% azide. Diluted antibody was divided as 4 x 2.5 ml in Falcon tubes. One 6 x 1 cm N/C strip carrying 50 µg of $p34^{cdc2}$ protein was put in a Falcon tube and rotated slowly on a wheel for at least 2h, or overnight, at room temperature. 400 µl of the original diluted antibody was taken as "initial antibody" for later comparison with purified fraction, to compare purity and determine yield.

3. Elution of purified antibody. The N/C strips were removed to a new Falcon tube and the residual liquid was kept as "unbound antibody". Each N/C strip was rinsed in 10 ml of 0.05% Tween20 TBS for 5 min. To each N/C strip, 1.6 ml of 0.2 M glycine pH 3.5 was added and washed over both surfaces of the N/C by hand for 2 min. The glycine was withdrawn and mixed with 300 µl of 1 M PO_4 pH 9.5 buffer to give pH 7.4 and the N/C strip was immediately put into a dish of water. 1 ml of 10% BSA and 0.5 ml of 3 M NaCl were added to 10 ml of the neutralised glycine eluate. This affinity purified antibody was dialysed at 4°C against 2 l of TBS containing 0.05% azide. The N/C strips were rinsed in H_20 and blocked in antibody blocking solution for 10 min, and rinsed 2 x 10 min in 0.05% Tween 20 TBS then rinsed in H_20 and dried for re-use. The affinity purified PSTAIR antibody was used with ^{125}I second antibody to quantify $p34^{cdc2}$-like protein in extracts of tobacco cells and pith tissue.

2.2.10. Purification of $p13^{suc1}$ protein

1. Induction of $p13^{suc1}$ by IPTG

The bacterial strain BL21(DE3), which contains a plasmid in which expression of the *suc1* DNA fragment from *S. pombe* is driven by the T7 promoter, was used according to the strategy of F W Studier and B A Moffat (1986) to overexpress $p13^{suc1}$ following induction of the T7 RNA polymerase with isopropyl-B-D-thiogalactoside (IPTG).

The bacterium was grown in a small scale LB medium culture containing 100 μ g/ml of ampicillin overnight and then transferred to a larger culture also containing 100 μ g/ml of ampicillin. IPTG was added when the OD_{600} of the culture was 0.4-0.5 to induce expresssion of $p13^{suc1}$ protein.

After the inducement with IPTG for 3h the cells were harvested by spinning for 5 min at 6,000g. The cells were resuspended in cold 20 mM tris-HCl pH 7.5. Glycerol was added to this cell suspension giving 15% final concentration. The cell suspension was then ready for storage by freezing or for purification of $p13^{suc1}$ protein.

2. Isolation and purification of $p13^{suc1}$ protein

The method was essentially as described by Brizuela et al (1987) with the addition of a phenyl Sepharose chromatography step.

(1). The cell suspension was supplemented with PMSF to 1 mM and cells were broken with a French press at 0°C.

(2). Some bacterial proteins were discarded by heating the extract to 70°C for 5 min by shaking in a water bath, then sedimenting denatured proteins at 100,000g for 15 min 4°C.

(3). The $p13^{suc1}$ protein in the supernatant was concentrated by precipitation at 70% saturated ammonium sulphate. 44.2 g ammonium sulphate was dissolved in 100 ml of supernatant, kept in ice for 1 hour and then centrifuged at 10,000 rpm in a JA 20 rotor for 15 min. The protein pellets were dissolved in less than 10 ml of 50 mM tris-HCl pH 7.5 and spun again at 10,000 g for 10 min to sediment any undissolved particles.

(4). The $p13^{suc1}$ supernatant was loaded onto a G75 Sephadex (Pharmacia), or S200 acrylamide, gel beads column that had been equilibrated with at least one bed

volume of 50 mM Tris-HCl pH 7.5. The column was eluted by one bed volume of the same buffer. The fractions containing $p13^{suc1}$ were identified by electrophoresis and pooled.

(5). For phenyl sepharose chromotography ammonium sulphate was added to 0.85 M by adding 11.3 g ammonium sulphate per 100 ml of $p13^{suc1}$ solution and the protein was loaded onto a phenyl Sepharose column that had been equilibrated with 0.85 M ammonium sulphate. The column was eluted by a declining concentration of ammonium sulphate from 0.85 M ammonium sulphate in 50 mM tris-HCl pH 7.5 to zero of the salt in the same buffer.

(6). The fractions which contain the prominent $p13^{suc1}$ protein were identified by OD_{280} and confirmed by 15% acrylamide gel electrophoresis. The protein was recovered by precipitation with 80% ammonium sulphate (52.3 g of ammonium sulphate added to 100 ml of $p13^{suc1}$) 4°C for 10 min and the pellet obtained at 10,000 g was dissolved in about 5 ml of PBS.

(7). The $p13^{suc1}$ protein in PBS was dialysed, using 3.5 kDa cut-off dialysis tubing (Spectrum Medical Industries, Inc), against 2 l of 25 mM HEPES pH 7.4 10 mM $MgCl_2$ 1 mM DTT buffer at 4°C overnight.

(8). The protein approximate concentration of $p13^{suc1}$ was determined by 1.55 A_{280} - 0.76 A_{260} and more accurately by Coomassie assay. The purity of $p13^{suc1}$ was determined by electrophoresis on a 15% acrylamide gel and purified $p13^{suc1}$ was used to make $p13^{suc1}$-Sepharose beads for purification of $p34^{cdc2}$-like protein kinase.

2.2.11. $p34^{cdc2}$-like protein kinase assay

The activity of $p34^{cdc2}$ and its homologues has been measured using α-casein or, much more effectively, H1 histone as substrate in yeast and animal cell extracts (Simanis and Nurse, 1986; Draetta and Beach, 1988; Booher et al., 1989; Gautier et al., 1989; Labbe et al., 1989; Roth et al., 1991; Rosenblatt et al., 1992) and also in extracts from higher plants (John et al., 1991; Feiler and Jacobs, 1991; Colasanti et al., 1991; Ferreira et al., 1991).

Prior to assay the enzyme was affinity purified on $p13^{suc1}$-beads and was eluted with free $p13^{suc1}$ in a variation of the method described by Blow and Nurse (1990).

2.2.11.1. Preparation of $p13^{suc1}$ beads

The $p13^{suc1}$ beads were prepered using Pharmacia cyanogen bromide Sepharose according to the manufacturer's instructions at a ratio of 8 mg of $p13^{suc1}$ to 1 ml of CNBr-Sepharose. After reaction with $p13^{suc1}$ the gel was resuspended in 10 ml of 1.0 M ethanolamine pH 8.0 and rotated on a wheel for 1h at room temperature to block unreacted Br groups, washed in 10 ml of 0.1 M PO_4 pH 7.5 three times and finally resuspended in equal volume of PBS with 0.01% merthiolate for storage at 4°C.

2.2.11.2. Purification of $p34^{cdc2}$-like protein kinase from cell suspension cultures of *N. plumbaginifolia* and from pith tissue or pith callus of tobacco

1. Buffers

(1). No detergent salt extract buffer (NDE). 20 ml of NDE buffer contained 800 µl of 500 mM HEPES pH 7.4, 2 ml of 1 M NaCl, 0.09 g DTT, 250 µl of 0.5 mM leupeptin, 100 µl of 100 mM of PMSF, 5 ml of 100 mM EGTA pH 7.3, 6.4 ml of 250 mM β-glycerophosphate, 200 µl of 100 mM sodium orthovanadate, 223 mg of disodium 4-nitrophenylphosphate, 2 ml of 500 mM sodium fluoride, 3 ml of 100 mM $MgCl_2$, 200 µl of 20 mM ammonium molybdate and 150 µl of H_20.

(2). High detergent wash buffer (HDW). 20 ml of HDW buffer contained 400 µl of 100 mM EDTA pH 7.2, 3 ml of 1 M NaCl, 1 ml of 20% of NP40, 417 µl of 0.5 mM leupeptin, 20 µl 100 mM of sodium orthovanadate, 2 ml of 500 mM sodium fluoride, 400 µl of 500 mM of Na-PO_4 buffer which is 300 mM Na_2HPO_4 and 200 mM NaH_2PO4, and 12.7 ml of H_2O.

(3). PBS-Mg-DTT buffer: PBS-Mg-DTT buffer consists of 2 ml of 10 x PBS, 2 ml of 100 mM $MgCl_2$, 3 mg DTT and 16 ml of H_20.

2. Purification of $p34^{cdc2}$-like protein kinase

(1). 0.1 g of powdered cells from suspension culture or (0.25 g of pith callus or pith tissue powder) was extracted with 300 µl (600 µl) of NDE buffer by 3 x 20 sec mixing on a vortex and the extract was centrifuged at full speed in Microfuge at 4°C for 5 min.

(2). 20 µl (30 µl for pith extract) of Sepharose 4B, which was rinsed in H_2O for twice and resuspended in equal volume of NDE buffer, was added to the 180 µl (350 µl of pith supernatant) of supernatant and the mixture was rotated for 1h on a wheel at 4°C, then centrifuged at full speed in a Microfuge at 4°C for 5 min.

(3). To the 190 µl (370 µl from pith extract) of supernatant was added 20 µl (or 30 µl for pith extract) of $p13^{suc1}$ beads. The mixture of the supernatant and $p13^{suc1}$ beads were rotated for 1 hour on a wheel at 4°C to bind $p34^{cdc2}$-like protein kinase to $p13^{suc1}$ beads then centrifuged at half speed in Microfuge at 4°C for 2 min.

(4). The $p34^{cdc2}$-$p13^{suc1}$ beads were rinsed in 400 µl of HDW buffer, spun down at half speed in Microfuge for 2 min and rinsed in 400 µl of HDW buffer again.

(5). The $p34^{cdc2}$-$p13^{suc1}$ beads were rinsed in 400 µl of PBS-Mg-DTT buffer and spun down at half speed in Microfuge for 2 min at 4°C.

(6). $p34^{cdc2}$-like protein kinase was eluted with free $p13^{suc1}$ by adding 50 µl of 0.5 mg/ml of $p13^{suc1}$ into the beads and rotating on a wheel for 30 min, then 45 µl of $p34^{cdc2}$-like protein kinase was collected in the supernatant by centrifuging at full speed in Microfuge for 5 min was kept in ice until assayed.

2.2.12. Assay of $p34^{cdc2}$-like protein kinase

Assay of $p34^{cdc2}$-like protein kinase based on the transfer of [^{32}P]ATP by $p34^{cdc2}$ protein kinase to dephosphorylated H1 histone or α-casein substrates.

(1). The reaction mixture for $p34^{cdc2}$-like protein kinase assay contained 25 mM β -glycerophosphate pH 7.3, 10 mM EGTA, 10 mM $MgCl_2$, 1 mM DTT, 25 mM HEPES pH 7.3, and per 50 µl assay 5 µg of H1 histone (or rarely α-casein), 100 pmole ATP and 0.25 µCi [^{32}P].

(2). 20 µl enzyme was added into the 30 µl of reaction mixture at 30°C in a water bath and the reaction was continued for 5 min until terminated by transfer to ice. Then

20 µl of reaction mixture was taken onto a piece of P81 phosphocellulose 10 x 20 mm and the paper was immediately put in 75 mM phosphoric acid to completely stop the reaction and remove free ATP.

(3). The remaining 30 µl of reaction mixture was mixed with an equal volume of SDS sample buffer (Sx2) and boiled for 3 min for fractionation by SDS-acrylamide gel.

(4). The P81 papers carrying 20 µl of reaction mixture were rinsed in 75 mM phosphoric acid 5 x 3 min and the bound radioactivity that had been transferred to protein was counted in a scintillation counter.

(5). The mixture of 30 µl of reaction mix with 30 µl of SDS sample buffer (Sx2) was separated on 0.75 mm thick 12% acrylamide gel. The gel was stained with fresh Coomassie solution which consists of 0.75 g G250, 338 ml of methanol, 75 ml of acetic acid and 338 ml of H_20. The stain was discarded to radioactive waste and the gel was washed and destained with a solution of 180 ml of methanol, 40 ml of acetic acid and 180 ml of H_20 for 1h, then again with a solution which consists of 40 ml of methanol, 60 ml of acetic acid and 700 ml of H_20.

(6). The gel was dried using the Biorad drier then photographed and the radioactive image was obtained by exposing the gel in a phosphorImager.

CHAPTER 3

CHANGES IN THE PROTEIN LEVEL AND CATALYTIC ACTIVITY OF $p34^{cdc2}$-LIKE PROTEIN KINASE IN THE DIVISION CYCLE OF TOBACCO (*NICOTIANA PLUMBAGINIFOLIA*) CELLS IN SUSPENSION CULTURE

3.1 Introduction

The cell cycle, as described in Chapter 1, can be divided into G1, S, G2 and M phases. A final cytokinetic phase at which the cytoplasm is separated into two daughter cells occurs in most cell cycles. Controls of progression from late G1 to S phase and from late G2 to M phase are very important in the cell division cycle. At the late G1 control point many cells make a choice between proliferation, quiescence or differentiation. They can enter the next division sequence, or abstain if their size or growth rate is inadequate or can divert metabolism from continued proliferation to differentiation. After the late G1 control point histones and enzymes catalysing deoxynucleotide and DNA synthesis are accumulated. During late G2 phase the $p34^{cdc2}$-cyclin B complex becomes catalytically active and cells undergo a series of changes to chromosome structure and a shift of cytoskeleton from cytoplasm to nucleus. However, the molecular basis of the control of transition from G1 to S phase and from G2 to M phase is not yet fully understood and is the subject of much current research.

3.1.1. Control at the G1 to S transition in yeast and animal cells

In *S. cerevisiae* progress from G1 to S is controlled by an event termed START, which can be viewed as the point of commitment to the mitotic cycle (Hartwell et al., 1974). Passage through the control point from G1 to S phase is dependent upon the absence of mating pheromones and upon the cell achieving a critical size (Hartwell et al., 1974; Reed, 1980). This START event has been shown by mutational analysis to involve the *CDC28* gene (Hartwell et al., 1974). The product of *CDC28* gene is a protein kinase (Lorincz and Reed, 1984). The active form of the cdc28 protein kinase was identified as

a complex of approximately 160 kDa containing an endogenous substrate, p40, and possibly other polypeptides (Mendenhall et al., 1987; Reed et al., 1985; Wittenberg and Reed, 1988) and it is now recognised that complex with proteins of the cyclin class is essential (Reed, 1991). Cells that arrest during G1 phase have inactive CDC 28 protein kinase that is not assembled into a complex. In late G1 phase activity of the protein kinase can be detected (Wittenberg and Reed, 1988) and is presumably involved in START. In the fission yeast *S. pombe*, a commitment control like START has also been located in G1. Its completion requires the *cdc*2 gene as indicated by mutational analysis (Nurse and Bissett, 1981; Booher and Beach, 1987). The first direct evidence for the molecular equivalence of eukaryote cell cycle control molecules came from the capacity of the budding yeast *CDC28* gene to complement lack of *cdc2* gene activity in fission yeast (Beach et al., 1982). However, there still is not any direct evidence of catalytic activity of $p34^{cdc2}$ at the late G1 to S transition in fission yeast although activity towards the heterologous substrate casein has been reported (Simanis and Nurse, 1986).

Most somatic mammalian cells also have a G1 phase that usually encompasses a major portion of the cell cycle. In many types of cell it has been observed that just before S phase cells acquire considerable resistance to inhibitors of protein synthesis in terms of commitment to initiate DNA replication, whereas prior to this point cells are extremely sensitive to such inhibitors (Rossow et al., 1979; Campisi et al., 1982; Zetterberg and Larsson, 1985). This point had been named the restriction point or R point (Pardee et al., 1974; Pardee, 1989) and it has been inferred from studies using inhibitors of protein synthesis that progression past the R point requires the accumulation of unstable proteins (Rossow et al., 1979; Campisi et al., 1982). The concept and properties of the R point have many similarities to START in budding yeast (Beach et al., 1982). Evidence indicating possible biochemical function of cdc2 at the G1/S transition has only recently emerged. In particular, it has been shown that cdc2 protein can phosphorylate SV40 T antigen, causing it to bind tightly to the SV40 T viral DNA origin of replication and significantly stimulate DNA replication in a cell-free system (McVey et al., 1989). Further evidence indicates that cdc2 stimulates DNA replication by phosphorylating

another protein, RPA, which is essential to initiation of DNA replication (Salah et al., 1990).

3.1.2. Control of the G2 to M transition in yeast and animal cells

A breakthrough in understanding the control of transition from G2 to M phase in eukaryotes resulted from analysis of conditional *cdc* mutants in yeasts. This genetical analysis has been supplemented by biochemical evidence of phosphorylation and dephosphorylation of $p34^{cdc2}$ and its association with binding proteins, particularly the cyclins, and by specific protein kinase and phosphatase, in particular those related to wee1 and cdc 25 (Nurse, 1990).

Fission yeast cells containing conditional mutations in the *cdc*2 gene could arrest in either late G1 or at the end of G2 if they were in G1 or G2 phases respectively at time of transfer to restrictive condition (Nurse and Bissett, 1981). The *cdc2* gene product $p34^{cdc2}$ is therefore required at two separate points in the cell cycle for entry into S phase and for entry into M phase. Homologues of $p34^{cdc2}$ have been diagnosed from their ability to replace *cdc2/CDC28* gene function in yeasts and have been detected in human cells (Lee and Nurse, 1987), mouse (Cisek and Corden, 1989) and chicken (Krek and Nigg, 1989), as well as in fruit flies (Lehner and O'Farrell, 1990). $p34^{cdc2}$ protein kinase is therefore considered a universal element in the control of the transition from G2 to M phase (Broek et al., 1991; Dunphy and Newport, 1988; Nurse, 1990).

3.1.3. Regulation of $p34^{cdc2}$ activity by phosphorylation and dephosphorylation

The reversible phosphorylation of specific residues in protein is a ubiquitous mechanism for the regulation of cellular processes. The level of $p34^{cdc2}$ protein may be constant throughout the cell cycle in cells that are continuing to proliferate, however, the activity of $p34^{cdc2}$ protein kinase changes up to 20 fold during the cell cycle. Evidence from yeasts and animal cells indicate that the change of activity of $p34^{cdc2}$ protein kinase is regulated by changes in its phosphorylation (Simanis and Nurse, 1986; Draetta and Beach, 1988; Gautier et al., 1989; Moreno et al., 1989). The results of mutating putative

phosphorylated residues to non-phosphorylatable residues and of comparing phosphopeptides derived from $p34^{cdc2}$ with synthetic peptides phosphorylated in *vitro,* as well as analysis of phosphoamino acids present in $p34^{cdc2}$, indicate that during most of G1 phase phosphorylation of $p34^{cdc2}$ is absent or at low levels. In chicken cells phosphorylation of serine (Ser 227) is reported in G1 phase (Krek and Nigg, 1991a) but has not been observed in other eukaryote cells. During G2 phase serine phosphorylation diminishes and phosphotyrosine (Tyr 15) and phospho-threonine (Thr 14 together with Thr 161 in chicken or Thr 167 in *S. pombe*) increases. The activity of $p34^{cdc2}$ is regulated by the change of phosphorylation state in these residues, especially in Thr 14 and Tyr 15, which lie within the deduced ATP binding site. Phospho-Thr 14 and phospho-Tyr 15 are completely dephosphorylated during prophase contributing to the activation of p34 protein kinase and establishment of M phase (Gould and Nurse, 1989; Nurse, 1990; Pondaven et al., 1990; Clarke and Karsenti, 1991; Krek and Nigg, 1991a; Krek and Nigg, 1991b; Yaacov et al., 1991; Lorca et al., 1992 and Fesquet et al., 1993).

Cdc 25 protein is required for activation of $p34^{cdc2}$ not only in fission yeast but also in *Xenopus,* human and probably all eukaryote cells (Russell and Nurse, 1986; Edgar and O'Farrell, 1989; Sadhu et al., 1990; Gould et al., 1990; Millar et al., 1991; Alphey et al., 1992). Biochemical evidence has indicated that cdc25 protein is a specific threonine tyrosine phosphatase that directly activates $p34^{cdc2}$ (Dunphy and Kumagai, 1991; Millar and Russell, 1992). The formula weight of the *cdc 25* gene product is 67 kDa but its mobility on SDS gels indicates an apparent size of 80 kDa and it is referred to as $p80^{cdc25}$. In fission yeast, the fact that levels of $p80^{cdc25}$ increase through G2, reaching a peak just before the onset of mitosis and that increased levels of $p80^{cdc25}$ due to additional copies of cdc 25 advance cells into mitosis suggest that the accumulation of $p80^{cdc25}$ to a critical level in the cell has a key role in determining the cell cycle timing of $p34^{cdc2}$ kinase activation in fission yeast (Russell and Nurse, 1986; Moreno et al., 1990). However in mammalian cells there are at least three homologues of cdc 25 and their levels can be relatively constant through the cell cycle. Therefore it is probably the activity of cdc25 proteins rather than the level of their protein that is critical. Activation of cdc25 catalytic activity in late G2 occurs by its phosphorylation (Hoffmann et al.,

1993) and may occur by cyclin binding since *cdc*25 phosphatase is specifically activated by B-type cyclin in human cells (Galaktionov and Beach, 1991). Homologues of cdc 25 have been conserved in evolution from yeast to animal cells and the contribution of cdc 25 to activation of $p34^{cdc2}$ by specific dephosphorylation of tyrosine 15, and in higher eukaryotes also threonine 14, is probably a universal eukaryote mechanism.

During the transition from the late G1 to S phase and the transition from G2 to M phase, it has been found that catalytically active $p34^{cdc2}$ and its homologues are in complexes with cyclins (Draetta et al., 1989; Pines and Hunter, 1991b; Reed, 1991; Xiong and Beach, 1991). In budding yeast the complexes were found to contain G1 cyclins (cln1, cln2 and cln3) and the active complexes are considered to regulate 'START' (Hadwiger et al., 1989b; Reed, 1991; Lew et al., 1992). In fission yeast $p56^{cdc13}$ (cyclin B) and $p34^{cdc2}$ interact closely at M phase (Hagan et al.,1988). A 45-kd component of purified MPF from *Xenopus* is a B cyclin (Labbe et al., 1989a; Gautier et al., 1990). It has been proposed that the function of cyclin B in mitotic phase is to facilitate the phosphorylation of $p34^{cdc2}$ that later releases catalytic activity when it is partially dephosphorylated at Thr 14 and Tyr 15 (Meijer et al., 1991; Parker et al., 1991).

3.1.4 Control points in cell division cycle in plants

The discovery of the control points in yeast cell cycles and increasing understanding of the molecular mechanisms in yeasts and animal cells has prompted investigation of the plant cell cycle. Some hypotheses have been put forward to account for division control in plants. For example, Van't Hof proposed a principal control point hypothesis, in which control points were proposed to occur in G1 and G2 phases rather than in S and M phases since meristematic cells arrest in G1 and G2 phases when they cease division (Van't Hof, 1974). In the unicellular green plant *Chlamydomonas* control in G1 phase was pinpointed to occur at an event that was functionally equivalent to the START control point. In the plant cell the late G1 control point is cell size dependent, marking a transition to cell cycle phases less dependent upon continued growth and committing a doubling of nuclear DNA to occur within 0.1 of a cell size (Donnan and John, 1983; John, 1984). These similarities with START prompted a search for a

$p34^{cdc2}$-like homologue in our laboratory and a $p34^{cdc2}$-like protein was found to be present and to show changes in level and phosphorylation that indicated cell cycle involvement (John et al., 1989). The molecular mechanisms of control points in the higher plant cell cycle have also begun to be understood as people have begun to pursue the possible presence of $p34^{cdc2}$-like protein kinase and cyclins in higher plant cells.

As described in Chapter 1, homologues of $p34^{cdc2}$ protein have been reported to be involved not only in the unicellular plant *Chlamydomonas*, but also in wheat, pea, alfalfa, maize and *Arabidopsis* (John et al, 1989; 1990; Feiler and Jacobs, 1990; Hirt et al, 1991; Ferreira et al., 1991). Further, homologues of the fission yeast *cdc*2 gene have been cloned by polymerase chain reaction or degenerate oligonucleotide probing from maize, pea, alfalfa and *Arabidopsis* (Colasanti et al., 1991; Feiler and Jacobs, 1991; Hirayama et al., 1991; Hirt et al., 1991). These results have been providing increasing evidence that homologues of $p34^{cdc2}$ are involved in the control of cell division cycle in higher plants.

3.1.5. Unresolved problems

Although there is evidence that $p34^{cdc2}$ protein kinase and its regulatory proteins are present in plant cells (John et al., 1989) there is little information concerning the possible control points of the higher plant cell cycle and the possible involvement of changes in $p34^{cdc2}$ kinase at these points. Therefore a suspension culture of *N. plumbaginifolia* was synchronised by aphidicolin block or nutrient limitation to explore control points in the division cycle and the changes of level and activity of $p34^{cdc2}$-like protein kinase in the cell division cycle of tobacco cells.

3.2 Materials and methods

3.2.1 Synchronisation of *N. plumbaginifolia* with aphidicolin

Aphidicolin at 5 mg/ml in DMSO was added for 24h to a suspension culture of *N. plumbaginifolia* previously grown for 12h after a standard dilution in fresh CSV medium that was routinely given 4 day intervals taking the cell density of about 6×10^6 per ml to

one sixth of that density. Cells that resumed growth after dilution were tested with final aphidicolin concentrations of 5 μg/ml, of 10 μg/ml or of 20 μg/ml. The cells, after treatment with aphidicolin for 24h, were spun down at 3,500 rpm for 2 min in a Clements 2000 centrifuge; washed twice by centrifugation with fresh CSV medium, resuspended in fresh CSV medium and sampled during the subsequent 24h for measurement of nuclear DNA content, rate of DNA synthesis indicated by incorporation of BrdU, level of $p34^{cdc2}$-like protein detected by PSTAIR antibody on Western blot and level of $p34^{cdc2}$-like protein kinase activity.

3.2.2. Nutrient limitation of cell-suspension cultures of *N. plumbaginifolia*

1. Suspension cultures of *N. plumbaginifolia* after growth for 4 days to 6 x 10^6 cells per ml were spun down at 3,500 rpm for 2 min, the cell pellet was collected and washed twice by centrifugation with nitrogen-free, phosphorous-free or sucrose-free CSV medium. Finally, cells were resuspended in different concentrations of nitrogen, phosphate or sucrose in CSV medium.

2. Media for nutrient limitation

(1). Nitrogen limiting CSV media

Nitrogen limiting CSV media were modified from standard CSV medium by reduction of NH_4NO_3 and omission of KNO_3. Supplementation with KCl and KH_2PO_4 maintained levels of P and K.

A. Nitrogen-free CSV medium

100 ml of nitrogen-free CSV medium contained 5 ml of 27.2 mM $CaCl_2.2H_2O$, 5 ml of 32.4 mM $MgSO_4.7H_2O$, 5 ml of 496 mM KCl, 5 ml of 80 mM KH_2PO_4, 100 μl of CSV Micro elements stock solution (1000 x), 100 μl of CSV Na_2EDTA stock solution (1000 x), 100 μl of CSV $FeSO_4.7H_2O$ stock solution (1000 x), 100 μl of CSV Organic stock solution (1000 x), 4.64 μl of 5 mM kinetin, 20 μl of 10 mg/ml 2,4-D, 0.1 g myo-inositol, 3 g sucrose, 30 ml of H_2O and pH 5.8.

Stock solutions are 1000 times final concentrations respectively.

B. Low nitrogen media

Media containing 2 mM or 4 mM nitrogen (1 mM or 2 mM NH_4NO_3) were comprised as in A with the addition respectively of 333 µl or 666 µl of 310 mM NH_4NO_3 per 100 ml prior to pH adjustment.

(2). Phosphate limiting CSV media

Phosphate limiting CSV media are the standard CSV medium modified by the change of $NH_4H_2PO_4$ amount.

A. Phosphate-free CSV medium

100 ml of no phosphate CSV medium contained 5 ml of 310 mM NH_4NO_3, 5 ml of 496 mM KNO_3, 5 ml of 27.2 mM $CaCl_2.2H_2O$, 5 ml of 32.4 mM $MgSO_4.7H_2O$, 100 µl of CSV Micro elements stock solution (1000 x), 100 µl of CSV Na_2EDTA stock solution (1000 x), 100 µl of CSV $FeSO_4.7H_2O$ stock solution (1000 x), 100 µl of CSV Organics stock solution (1000 x), 4.64 µl of 5 mM kinetin, 20 µl of 10 mg/ml 2,4-D, 0.1 g myo-inositol, 3 g sucrose, 30 ml of H_2O and pH 5.8.

B. Low phosphate CSV media

Low phosphate CSV media containing 0.1 mM and 0.25 mM phosphate were comprised as in A with the addition respectively of 125 µl and 250 µl of 80 mM $NH_4H_2PO_4$ per 100 ml prior to pH adjustment.

(3). Sucrose limiting CSV media

Sucrose levels were reduced to zero, 5 mM or 10 mM by addition of 0, 17.1 mg, or 34.1 mg per 100 ml and no adjustment of other nutrient compositions was necessary to maintain the balance of major nutrients.

3.2.3. Detection of DNA synthesis in suspension culture

1. Labelling cell-suspension culture with BrdU

0.5 ml of suspension culture was taken for labelling nuclei with bromo-deoxyuridine (BrdU). 5 µl of 100 mM BrdU was added giving a final concentration of 100 µM . This suspension was incubated with BrdU for 30 min under the same conditions as the main culture at 25°C and 100 rpm shaking. The cells after labelling

were spun down and rinsed twice by centrifugation in PBS. The cells were fixed in 4% (v/v) paraformaldehyde in PBS for 1h, and rinsed twice in PBS then were ready for immuno-detection of incorporated base analogues.

2. Immunofluorescent detection of BrdU-DNA

The cells were digested in 2% (w/v) cellulysin cellulase (Calbiochem 219466) in 0.4 M Mannitol PM5E buffer, which contains 50 mM PIPES, 5 mM EGTA, 2 mM $MgSO_4.7H_2O$ and 0.005% NaN_3, for 10 min and then permeabilised in 1% NP40 in PM5E buffer for 15 min after rinsing in PM5E buffer 2 x 3 min. After rinsing off NP40 in PM5E on the slide, the cells were incubated in 20-30 µl of undiluted first anti-BrdU antibody (Amersham) at 37°C for 3h or overnight. After rinsing off the first antibody the cells were incubated at 37°C for 1-2h with a FITC-conjugated sheep anti-mouse IgG antibody (SAM: Silenus Lab Ltd., Dandenong, Australia) diluted 1:30 in PBS with 1% (w/v) bovine serum albumin and 0.02% (w/v) NaN_3. After rinsing off the secondary antibody the cells were also stained with 0.2 µg/ml DAPI in PBS for 3-5 min at room temperature to locate nuclear DNA. The cells were mounted in glycerol-polyvinyl alcohol (Mowiol; Hoechst A.G., Frankfurt, FRG) containing 0.1% paraphenylenediamine.

Nuclei labelled with BrdU-associated fluorescence were detected by use of Zeiss Axioplan and Axiovert microscopes equipped with fluorescence optics and standard FITC filter set.

3.3. Results

3.3.1. Effects of aphidicolin on nuclear DNA content of cells in suspension culture

At present there is little evidence concerning molecular mechanisms at the late G1 to S transition and late G2 to M transition in plant cells, because population of cells in tissues are in different cycle phases. Cell suspension cultures provide a useful way to

study the control in the division cycle because cells can be synchronised by arrest in specific phases using chemical or nutrient treatments.

Aphidicolin, which inhibits DNA polymerases in eukaryotic cells from yeast to animals (Ikegami et al., 1978; Pedrail-Noy and Spadari, 1979), was used to treat suspension cultures of *N. plumbaginifolia*. Cells which had grown for about 12h after standard dilution in fresh CSV medium were supplemented with aphidicolin to 5, 10 or 20 μg/ml and then allowed to grow for 24h. The cells after release from the aphidicolin block were taken for the observation of change of nuclear DNA content. Normal proliferating cells and cells that were treated with 5 μg/ml of aphidicolin for 24h when analysis for frequency of nuclear DNA content showed two peaks, one at about 55-60 relative units, the other at 110-120 relative units, and the frequency distributions of nuclear DNA content are the same for both (Fig. 3.1.a-b). However, the frequency of nuclear DNA content shows only one peak, centered on 60 relative units, when cells were treated in 10 μg/ml or 20 μg/ml of aphidicolin for 24h (Fig. 3.1. c-d). These results imply that about 55-60 relative units are the fluorescence intensity of nuclear DNA in G1 phase and 110-120 relative units indicate the fluorescence intensity of nuclear DNA in G2 phase. Cells treated with more than 10 μg/ml of aphidicolin for 24h were arrested at the beginning of S phase. The peak of nuclear DNA content moved to 110-120 relative units (G2 phase) when cells grew for 16h after release from the aphidicolin block and then as the population passed through mitosis back to about 60 relative units (G1 phase) when cells grew on to 24h (Fig. 3.2.a-c). The recovery of DNA synthesis of nuclei after release from the aphidicolin block means that the effects of aphidicolin on DNA polymerases is temporary and the DNA polymerases still functioned after release from inhibition by aphidicolin.

3.3.2. Effects of aphidicolin on cell number density

A suspension culture which had grown asynchronously for 12h after dilution in fresh CSV medium was blocked with 10 μg/ml or 20 μg/ml of aphidicolin for 24h, spun down and rinsed three times by centrifugation in CSV medium and then resuspended in fresh CSV medium. Samples were taken during the first 24h after release from the

aphidicolin block for measurement of cell number density and detection of DNA synthesis by incubation of a subculture with BrdU. Nuclei of the cells were stained with DAPI and cell number density was counted with haemocytometer. Fig. 3.4.a shows the change of cell number density and of nuclei labelled with BrdU from 0 to 24h after released from aphidicolin. Cell number density did not change greatly from 0 to 17h, but nearly doubled by 22h. Cells after released from aphidicolin block therefore divided in about 20-22h. Cells labelled with BrdU showed a sharp peak of incorporation in which about 50% of cells were labelled with BrdU at 1h and declined to a near constant based level of 6 % from 10h onwards.

These data therefore indicate that DNA synthesis in tobacco cells can be blocked by inhibiting DNA polymerases with aphidicolin and that cells can synchronously resume cycling by progressing through S phase between 0-4h after removal of the inhibitor, residing in G2 phase between 4 and 17 h and then carrying out mitosis, leading to a doubling in cell number between 20 and 22 h.

3.3.3. Effects of limiting nitrogen, phosphate and sucrose on the cell number in asynchronous cultures of *N. plumbaginifolia*

Many eukaryote cells arrest in a specific phase of the cell cycle when their growth rates decline. Budding yeast cells arrest in G1 phase (Pringle, 1975), fission yeast in G1 or G2 phase (Nurse, 1975) and nonmalignant animal cells in low-serum medium frequently arrest in G1 phase (Pardee et al., 1974). Higher plant cells have not been systematically studied in this respect therefore in the present study nitrogen, phosphate and sucrose were limited to investigate the possibility that cells might arrest in specific phases of the division cycle.

An asynchronous suspension culture that had been cultivated for 4 days was spun down and washed twice by centrifugation in nitrogen-free, phosphate-free or sucrose-free CSV medium at the same cell number density. Washed cells were transferred into different concentrations of nitrogen, phosphate or sucrose CSV media. Cells were taken for the count of cell number density at different time points. Nuclei were stained with DAPI and counted with a Nikon microscope and haemocytometer.

Cells which were transferred into nitrogen-free CSV medium could continue to divide during the first day, presumably utilising carried over nitrogen, however they could not perform a second division. Cells which were transferred into 2 mM or 4 mM nitrogen CSV medium continued division until the second and third days (Fig. 3.6.a). A linear response to nitrogen in the 0 to 2 mM range was indicated by the stationary cell number observed at day 4 (Fig. 3.6.a). Similarly the final cell number densities in limiting phosphorous media indicated a linear response in the range 0 to 0.25 mM but some cell lysis may have occurred after the fourth day in all limited cultures. (Fig. 3.7. a). Also the final cell number densities in limiting sucrose mediua indicated a linear response in the range 0 to 10 mM but some cell lysis may have occurred after the second day in 0 and 5 mM sucrose limited cultures (Fig. 3.8. a).

Cell division in suspension cultures of *N. plumbaginifolia* can clearly be limited by low nitrogen, phosphate or sucrose in CSV medium. It was therefore appropriate to consider whether arrest had occurred in a particular cell cycle phase.

3.3.4. Effects of limiting nitrogen, phosphate or sucrose on nuclear DNA content

Cells which grew in nitrogen-free, phosphate-free or sucrose-free CSV medium for 4 days were taken for the measurement of nuclear DNA content by staining with propidium iodide.

The distribution of frequency of nuclear fluorescence intensity of cells which grew in sucrose-free CSV medium for 4 days indicates that a majority of cells are in G2 phase because the peak of the distribution was at about 110 relative units (Fig. 3.8.b). Whereas the distribution of frequency of nuclear fluorescence intensity of cells which grew in nitrogen-free or phosphate-free CSV medium for 4 days coincides with the distribution of nuclear DNA fluorescence intensity in G1 phase (Fig. 3.6.b and Fig. 3.7.b). Therefore arrest in either G1 or G2 phase can be obtained by nutrient limitation.

Arrest in a specific cycle phase was confirmed by the DNA synthesis patterns of cells which were transferred into complete CSV medium after release from starvation for nitrogen, phosphate and sucrose. After release from nitrogen starvation, which induced

G1 phase arrest (Fig. 3.6.a-b), DNA synthesis peaked at 6h after transferring cells from nitrogen-free CSV medium into complete CSV medium as detected by labelling cells with BrdU (Fig. 3.9.d). This correlates with evidence of an initially predominantly G1 nuclear DNA content at 0h and its increase to G2 levels. Similarly cells transferred from phosphate starvation into complete CSV medium were initially in G1 phase (Fig. 3.12.a) and were labelled with BrdU predominantly during the first 12h, which correlated with an increase in DNA content to early G2 levels around 15h (Fig. 3.12.b). Whereas cells transferred from sucrose starvation, which were in G2 phase, were scheduled to enter mitosis and then G1 phase in the next cycle before entering S phase indicated by the change of nuclear DNA content in which the nuclear DNA content of most cells reached that of G1 phase at 24h (Fig. 3.15.a-c) and continued to progress in S phase at 27h as indicated by labelling nuclei with BrdU (Fig. 3.15d).

Novel means of obtaining synchrony were therefore established in higher plant cell suspension cultures and these were exploited for the study of cell cycle control molecules.

3.3.5. $p34^{cdc2}$-like protein level in the cell cycle of *N. plumbaginifolia* studied after synchronisation by aphidicolin inhibition or nutrient limitation

The level of $p34^{cdc2}$ protein during the higher plant cell cycle is of interest since several alternative patterns have been reported in other cell types, as will be detailed in the discussion of this section. To detect possible side effects of synchronisation, which cannot easily be identified if a single means of synchronisation is employed, cells were studied on resumption of cycling after aphidicolin inhibition and also after nutrient limitation.

Cells which were predominantly synchronised in early S phase were obtained by a block with 20 µg/ml of aphidicolin for 24h and were studied after washing twice in fresh CSV medium and transfer into fresh CSV medium. A sharp peak of DNA synthesis at 1h after release from the aphidicolin block, revealed by incorporation of BrdU, indicated that arrested cells were predominantly in S phase (Fig. 3.4.a). Cell number density

doubled after 21h and implied that cells had previously passed G2 and M phases (Fig. 3.4.a). Samples for measurement of $p34^{cdc2}$-like protein, were taken every two hours between 0 and 24h after release from the aphidicolin block. Proteins were extracted into RIPA buffer and run on 10-15 % linear gradient acrylamide gel and then transferred to 0.45 micron nitrocellulose (Amersham). Fig. 3.3 shows $p34^{cdc2}$-like protein was detected in the blot using antibody raised against the EGVPSTAIREISLLKE sequence (PSTAIR peptide), which has been perfectly conserved in $p34^{cdc2}$ between yeasts and humans (Lee and Nurse, 1987) and in some closely related *cdc2*-like proteins but not in other protein kinases (Hanks et al., 1988). The antibody used here has detected $p34^{cdc2}$ in yeasts and animal cells (Lee and Nurse, 1987; Gautier et al., 1988) and a homologous protein that is involved in the cell cycle of the unicellular plant *Chlamydomonas*, pea, wheat and *Arabidopsis* in our laboratory. The antibody recognised a tobacco protein of size 34 kDa after affinity purification by binding to mammalian $p34^{cdc2}$. Recognition was specific for the conserved PSTAIR region because it was eliminated by competition with 20 nM PSTAIR peptide (Fig. 3.3).

Fig. 3.4.b-c shows the change in level of $p34^{cdc2}$-like protein detected with the first antibody against PSTAIR, and ^{125}I anti-rabbit-IgG as the second antibody, in cells from 0 to 24h after release from the aphidicolin block. During this period cells progressed through S phase and then G2 and M phases. Fig. 3.4.c is a phosphorImage of $p34^{cdc2}$-like protein from 0 to 24h after release from the aphidicolin block. Fig. 3.4.b quantifies the change in level of $p34^{cdc2}$-like protein from 0 to 24h after release from the aphidicolin block. These results show that the level of $p34^{cdc2}$-like protein does not change greatly from S phase to M phase in the division cycle of suspension cultured of *N. plumbaginifolia* cells, but data for G1 phase are limited by the declining synchrony in later samples. Alternative means of synchronisation were therefore employed.

Information concerning the level of $p34^{cdc2}$ -like protein during progress through G1 to S, G2 and M phases can be obtained from cells which were blocked in G1 phase by nitrogen starvation or phosphate starvation for 4 days and then transferred to complete CSV medium to grow for 33h. Cells which were in late G1 phase after nitrogen starvation can be characterised in cycle progression after transfer into complete

CSV medium by a change in nuclear DNA content and labelling with BrdU. In Fig. 3.9.a-c, a change in the distribution of nuclear DNA content shows synchronous cells which were in late G1 phase after nitrogen starvation for 4 days had progressed to G2 phase after transfer into complete CSV medium for about 24h. Synchronous entry into S phase early in the period of resumed growth was confirmed by a change in the percentage of cells labelled with BrdU (Fig. 3.9.d), which peaked at 6h after release from nitrogen starvation. $p34^{cdc2}$-like protein in these samples was detected using antibody against PSTAIR and ^{125}I second antibody. Fig. 3.10.b shows a phosphorImage of $p34^{cdc2}$-like protein in 50 μg samples of total protein from cells sampled between 0 and 33h after release from nitrogen starvation. Fig. 3.10.a shows a quantification of the level. These results show that the level of $p34^{cdc2}$-like protein, as a proportion of total extractable protein, in cells which were blocked with nitrogen starvation in late G1 phase and then transferred into fresh CSV medium to grow for 33h to mitotic phase, gradually increased from 0 to 33h after release from nitrogen starvation but no more abrupt increase in the level of the enzyme protein was detected at G1/S or G2/M boundaries, when cdc2 is expected to act. It is possible that when cells were previously nitrogen starved proteins were turned over and there may have been selective less of the division protein, which has no known function in starved cells. On restoration of general protein synthesis the level of $p34^{cdc2}$-like protein may have been gradually restored. This interpretation can be tested by measuring the level of $p34^{cdc2}$-like protein in cells resuming cycling after phosphate starvation, which is not expected to enforce turnover.

Fig. 3.12 shows the change of nuclear DNA content in cells which were blocked by phosphate starvation in CSV medium for 4 days and then transferred into complete CSV medium for 33h. After phosphate starvation cells were arrested in G1 phase (Fig. 3.12.a) and had progressed to G2 phase after transfer to complete CSV medium for 30h (Fig. 3.12.c). Labelling nuclei with BrdU confirmed that the cells which were arrested in G1 phase had progressed through S phase by about 15h when the nuclear DNA content profile confirmed a majority of cells in G2 phase. The level of $p34^{cdc2}$-like protein in these cells after release from phosphate starvation was not seen to change greatly in samples of protein extracted in RIPA buffer and separated on a 10-15 % linear gradient

acrylamide gel. Equal protein loadings for each sample were transferred onto nitrocellulose and then detected with antibody against PSTAIR peptide as the first antibody and ^{125}I-anti-rabbit IgG as the second antibody (Fig.3.13. a-b). The similarity of pattern is significant since these cells under phosphate limitation stress are under different physiological conditions from aphidicolin blocked cells that are able to continue in general metabolism and growth and also differ from nitrogen limited cells that were recovering from stress in an entirely different area of metabolism.

Previously described experiments employed cells that were resuming the cell cycle in G1 or S phases. To investigate the accumulation of p34^{cdc2}-like protein in cells resuming in G2 phase the cell cycle after sucrose starvation was studied. Cells were arrested in G2 phase after sucrose starvation for 4 days, as shown by the distribution of nuclear DNA content of cells (Fig. 3.15.a). On release from starvation cells recovered slowly and remained in G2 until, at least 15h, from the evidence of nuclear DNA content, but then passed through mitosis and by 24h had reached G1 phase of the next cell cycle (Fig. 3.15.b-d). Fig. 3.16.b shows the phosphorImage of p34^{cdc2}-like protein during 33h after release from sucrose starvation and the level is quantified in Fig. 3.16.a. The essentially constant level further confirms that changes in the level of p34^{cdc2}-like protein are unnecessary for progress through the cell cycle and are not present in cells recovering from DNA block or from nutrient limitation. The possibility is not eliminated that developmental stimuli such as changes in hormone level, which may induce cessation of division, could lower p34^{cdc2}-like protein level to a point at which synthesis in specific cell cycle phases could be necessary. Such synthesis of p34^{cdc2}-like protein on hormone stimulation of resumed division is described in Chapter 6.

3.3.6. Quantitative recovery of p34^{cdc2}-like protein kinase by p13^{suc1} binding for assay of activity

In previously studied eukaryote cells, the activity of p34^{cdc2} protein kinase and of its homologues has been measured in *vitro* using histone H1 and sometimes other substrates. Activity of p34^{cdc2} protein kinase has been reported at START, the late G1/S transition, and especially at mitosis in yeast, frog and human cells. In the present

study cells which were in late G2 and M phase, exhibiting a maximum of $p34^{cdc2}$-like kinase activity after release from aphidicolin block were used to test the recovery of $p34^{cdc2}$-like protein kinase. $p34^{cdc2}$-like protein kinase was isolated and purified from cell powder in NDE buffer using $p13^{suc1}$ beads and eluting with free $p13^{suc1}$. 20 µl of $p13^{suc1}$ beads (8 mg protein per ml of gel) were used to couple $p34^{cdc2}$-like protein kinase and 50 µl of 0.5 mg/ml of $p13^{suc1}$ solution were used to selectively elute the kinase beads. The activity of $p34^{cdc2}$-like protein kinase was measured at 30°C for 5 min using histone H1 as substrate (Fig. 3.5.b). The recovered activity of $p34^{cdc2}$-like protein kinase increased linearly as the amount of cell material increased from 0.016g to 0.128g. In subsequent experiments the amounts of cell material were kept below 0.128g to obtain linear recovery.

3.3.7. Activity of $p34^{cdc2}$-like protein kinase in the cell cycle of *N. plumbaginifolia* synchronised by aphidicolin or nutrient limitation

Although the level of $p34^{cdc2}$ protein need not change to allow progress through the cell cycle, data from other eukaryotes indicated that changes in catalytic activity may be involved at key times, especially during mitosis. To obtain such data from the higher plant cell cycle, measurements of catalytic activity were made in cells that were synchronised by blocking with aphidicolin and by starvation for nitrogen, phosphorous or carbon.

3.3.7.1. Synchronisation with aphidicolin

Fig. 3.5.a shows the change of $p34^{cdc2}$-like protein kinase in the cells which, prior to sampling, were blocked with 20 µg/ml aphidicolin for 24h, washed twice by centrifugation in CSV medium and transferred into fresh CSV medium for a 24h sampling period. The cells were arrested prior to S phase after blocking for 24h, as shown by the initial nuclear DNA content indication of G1 phase (Fig. 3.2.a). An early progress through S phase was confirmed by high frequency of BrdU incorporation into nuclear DNA peaking at 1h (Fig. 3.4.a) In this culture the peak of activity of $p34^{cdc2}$-like protein kinase was at 19h after release from the aphidicolin block (Fig. 3.5.a) and

coincided with mitosis since it preceded by 2h the time of doubling in cell number that indicates the completion of division. Presence of detectable activity of $p34^{cdc2}$-like protein kinase from 0 to 15h after release from the aphidicolin block and prior to M phase in most cells is most simply explained by incomplete synchrony that is further reflected in the maximum incidence of DNA synthesis at any one time of 50 %.

3.3.7.2. Synchronisation by nutrient starvation

Nitrogen starvation for 4 days blocked cells in late G1 phase as shown by their nuclear DNA content of around 60 units (Fig. 3.9.a). The G1 phase arrest was confirmed by the incorporation of BrdU into about 75 % of nuclei DNA at 6h after transfer into complete CSV medium (Fig. 3.9.d). The cells progressed through S phase and attained in late G2 phase by 30h, shown by the predominance of nuclear DNA contents around 110 units (Fig. 3.9.c). In this culture the peak of activity of $p34^{cdc2}$-like protein kinase when histone H1 was used as substrate, was at 33h after release from nitrogen starvation and coincides with nuclei in late G2 phase entering mitosis (Fig. 3.11.b). Conversely a peak in the activity of $p34^{cdc2}$-like protein kinase, when α-casein was used as substrate occurred at 0h after transfer into complete CSV medium (Fig. 3.11.a) and coincides with the presence of nuclei in G1 phase containing around 60-70 units (Fig. 3.9.a). The α-casein kinase activity also coincided with the peak of incorporation of BrdU into DNA (Fig. 3.9.d) and is consistent with this being an S phase activity.

Phosphate starvation arrested cells in early G1 phase as shown by nuclear DNA contents of 50 units (Fig. 3.12.a) and by 30h this had doubled (Fig. 3.12.c) indicating that G2 phase had been attained. This arrest in G1 phase and subsequent progress through S phase was confirmed by the incorporation of BrdU into nuclear DNA during the first 12h after release from phosphate starvation (Fig. 3.12.d). In this culture the peak of activity of $p34^{cdc2}$-like protein kinase, when histone H1 was used as a substrate, occurred at 27h after release from phosphate starvation and coincides with presence of nuclei in late G2 phase (Fig. 3.12.c). However, no obvious activity of $p34^{cdc2}$-like protein kinase could be detected from 0 to 33h after transfer into complete CSV medium

when α-casein was used as substrate (Fig. 3.14.a), although cells progressed through S phase and attained late G2 phase (Fig. 3.12.a-c).

Sucrose starvation arrested a proportion of cells in early G2 phase, as shown by nuclear DNA contents around 110 units (Fig. 3.15 a); and these cells only slowly resumed cycling, remaining in G2 until at least 15h, as indicated by nuclear DNA contents (Fig. 3.15.a-b). Subsequent progress through M phase is indicated by attainment of G1 phase in the next cycle by 24h (Fig. 3.15.c), and the subsequent S phase at 27h after release from sucrose starvation was detected by incorporation of BrdU into nuclear DNA (Fig. 3.15.d). In this culture the peak of activity of $p34^{cdc2}$-like histone kinase was at 21h when histone H1 was used as a substrate after release from sucrose starvation (Fig.3.17.b). When α-casein was used as a substrate no obvious activity of $p34^{cdc2}$-like protein kinase could be detected from 0 to 33h after release from sucrose starvation (Fig. 3.17.a) although cells progressed through M phase and attained G1 phase at 24h after release from sucrose starvation (Fig. 3.15.a-c). However, synchrony was poor after sucrose starvation and extremely poor by the time S phase was attained late in the experiment.

Activity of $p34^{cdc2}$-like protein kinase could be detected, with histone H1 as a substrate, in cells which were in late G2 to early M phase, when suspension cultures were sychronised by either aphidicolin inhibition or by starvation for nitrogen, phosphate or sucrose. When α-casein was used as a substrate protein kinase was detected in the protein fraction, which was affinity purified by $p13^{suc1}$ binding from cells that were in late G1 and S phase, following synchronisation by nitrogen limitation. This activity was not detected in cells synchronised by phosphate or sucrose limitation which gave poorer synchrony of S phase.

3.4. Discussion

3.4.1. Synchronisation of division in suspension culture cells

To apply biochemical analysis to events of the cell cycle it is necessary to obtain sufficient material that is representative of cells in different phases of division. For most

analyses this requires synchronisation of division in cell populations. Synchrony is obtained with minimum disruption of cell physiology by selecting cells on the basis of cell size for further culture or for immediate analysis. This method is particularly suitable for yeast cells, which are regular in morphology and couple division precisely to the attainment of critical cell size (Fantes and Nurse, 1981) but selection is also useful with mammalian cells (Draetta et al., 1987). Unfortunately even the long-established cell lines of higher plants tend to form cell clumps that prevent the selection of individual cells of defined size.

It is therefore necessary to obtain synchrony of plant cell division by the alternative means of blocking an essential cell cycle event such as DNA synthesis. Use of an inhibitor to obtain such a block has the disadvantage that cells can continue growth and become unusually large. Experiments based entirely on use of a single inhibitor have two additional disadvantages. First, the inhibitor may have side effects on general metabolism that may not be detected because they are always present with the inhibitor. Second, arrest at a single cell cycle point means that synchrony will always have decayed by the time the population has progressed to the cycle phase most remote from the block. The present study therefore attempted additional means of synchronising cell populations other than by use of a DNA synthesis inhibitor and was able to obtain arrest in G1 phase by limiting either nitrogen or phosphorous, and also arrest in G2 phase by limiting sucrose. This diversity of procedures, which are expected to impose entirely different forms of physiological stress, reduces the possibility that a phenomenon that derives from physiological stress will be wrongly interpreted as part of the cell cycle.

Preferential arrest in specific cycle phases due to limiting macronutrients which was observed in suspension cultured cells is of interest in relation to the earlier observation by Van't Hof (1974) that differentiating cells in tissue do not arrest in S or M phases but rather in G1 or G2 phases. Van't Hof proposed that principle control points were located in G1 or G2 phase but no data were available concerning their number or their location relative to the initiation of S phase or M phase. Van't Hof considered that changes in hormone level in whole tissues may have caused the arrest of cell cycle progress and initiation of differentiation. The present data were obtained with constant

levels of externally supplied hormone and indicate that changes in nutrient availability are also registered by cell cycle modulators that act in G1 or G2 phases.

3.4.2. Levels of $p34^{cdc2}$-like protein in the cell cycle of a higher plant

This investigation has for the first time in any plant cell type provided data concerning the level of $p34^{cdc2}$-like protein through the cell cycle. There is a consensus of evidence from the suspension culture cells studied here that the protein kinase was held at a relatively constant ratio to other cell proteins throughout the cell cycle. This was seen in cells previously subjected to nitrogen starvation (Fig. 3.10) or phosphate starvation (Fig. 3.13), both of which resulted in a cell cycle beginning in G1 phase (Fig. 3.9. and 3.12) but different physiological stresses. A constant level of $p34^{cdc2}$-like protein was maintained by cells resuming after aphidicolin treatment (Fig. 3.4), which, unlike nutrient limitation, does not prevent growth and results in resumption of the cell cycle in S phase (Figs. 3.2.a-c 3.4). A constant level was also seen in cells resuming after sucrose starvation (Fig. 3.16); which presumably improses a stress peculiar to carbon starvation and results in resumption in G2 phase (Fig. 3.15). The cells recovering from nitrogen starvation did show a steady slow increase in $p34^{cdc2}$-like protein level (Fig. 3.10.a) that did not correlate clearly with any cell cycle events and may present a restoration of level previously reduced by starvation. The maintenance of a stable level of $p34^{cdc2}$-like protein relative to total extractable proteins is therefore a phenomenon that occurs under a wide range of physiological conditions in cells that are in suspension culture and not receiving developmental signals because in constant supplied levels of 2,4-D and kinetin.

The finding of relatively constant levels of $p34^{cdc2}$-like protein in the plant cells correlates with the evidence from proliferating yeast cells (Lorincz and Reed, 1984; Simanis and Nurse, 1986) and human cancer cells (Draetta and Beach, 1988). We cannot eliminate the possibility that there may have been minor changes in rate of synthesis since the measurement of total amount of $p34^{cdc2}$-like protein is an insensitive method for detecting minor fluctuation in the rate of synthesis. Also we cannot eliminate the possibility that variants of $p34^{cdc2}$, perhaps equivalent to CDK2 in animal cells

(Elledge and Spottswood, 1991), contribute to different extents to the total signal for PSTAIR protein, although it would be remarkable if such fluctuation happened to be exactly reciprocal. The possibility that there might be minor changes in rate of synthesis has some precedence. In human cells, at least one laboratory (McGowan et al., 1990) have reported fluctuation in cdc2 mRNA level and have pointed out that if newly synthesised $p34^{cdc2}$ has special properties then an increased rate of synthesis will have a bigger effect than its small change in the total $p34^{cdc2}$ protein might indicate. It will be interesting to test the levels of cdc2 mRNA through the plant cell cycle when a readily synchronised cell type is available of a plant species from which the cdc2 gene has been cloned.

Although significant changes in $p34^{cdc2}$-like protein level were not found in the synchronous cultures it should be noted that the gene can be strongly regulated during normal development of multicellular animals, and in plants following the application of phytohormones to plant tissues. In wheat leaves, accumulation of $p34^{cdc2}$-like protein is maximal in dividing cells at the basal meristem and ceases during subsequent cell differentiation, in which cell growth and expansion cause $p34^{cdc2}$-like protein to decline to one-sixteenth of the level in dividing cells (John et al., 1990). $p34^{cdc2}$-like protein is at a maximal level during the cell division phase of carrot cotyledon development and it declines sharply as cells switch to the differentiation program of enlargement and photosynthesis. In this dicotyledonous carrot tissue, in which resumption of division can be induced, a positive correlation of increase in $p34^{cdc2}$ protein with resumption of division was seen in excised segments transferred to 2,4-D medium (Gorst et al., 1991). In the present thesis a more extensive study of hormone mixtures confirm this correlation (Chapter 6).

Although $p34^{cdc2}$-like protein was maintained at a constant level throughout the cell cycle the activity of $p34^{cdc2}$-like protein kinase was restricted to certain phases, as will be discussed in the next section.

3.4.3. Change of activity of $p34^{cdc2}$-like protein kinase during the division cycle of *N. plumbaginifolia* cells in suspension culture

Activity of $p34^{cdc2}$ -like protein kinase was detected after affinity purification using the $p34^{cdc2}$ binding protein $p13^{suc1}$. Proteins that were specifically bound to $p13^{suc1}$, which was covalently coupled to Sepharose beads ($p13^{suc1}$ beads), were eluted by displacement with free $p13^{suc1}$. The elution step was found in oocyte extracts to give a considerable increase in purity over the heterogenous protein fraction that bound to $p13^{suc1}$ beads (Blow and Nurse, 1990). Although this activity is expected to be predominantly that of $p34^{cdc2}$ we cannot eliminate the possibility that close variants of $p34^{cdc2}$ that also bind to $p13^{suc1}$, such as CDK2 (Fang and Newport, 1991; Pagano et al., 1992) may have contribute to the activity that was measured. The number and role of $p34^{cdc2}$-related proteins is a particularly uncertain area at time of writing and no data are available concerning their certain presence biochemical properties in plants. To date there is no evidence that any $p34^{cdc2}$ variant that binds to $p13^{suc1}$ has a function other than in the cell cycle. Since $p34^{cdc2}$ is the most abundant cell cycle kinase (Booher et al., 1989; Moreno et al., 1989) the activity measured here is presumably mostly $p34^{cdc2}$ and it was analysed by currently the best available method.

A mitotic peak of $p34^{cdc2}$-like protein kinase activity, measured by phosphorylation of H1 histone that is probably a natural mitotic substrate (Langan et al., 1989), was detected in cells synchronised by all four methods. In aphidicolin-synchronised culture, cells commenced growth in late G1 phase as indicated by nuclear DNA content (Fig. 3.1) rapidly entered S phase detected by BrdU incorporation (Fig. 3.4.a) and completed S phase within 6h. At 16h cells in G2 phase began to show an increase in $p34^{cdc2}$-like H1 kinase activity (Fig. 3.5.a) that coincided with an increasing incidence of mitotic activity that resulted in an increase in cell number from 19h (Fig. 3.4.a) following the peak in kinase activity. There is evidence that synchronisation by aphidicolin was incomplete since a basal level of BrdU incorporation and of cell number increase was detected throughout the sampling period (Fig. 3.4.a), therefore the basal level of H1 kinase can most simply be explained as due to the presence of mitotic cells that are out of synchrony with the majority of the population.

The mitotic H1 histone kinase activity in the $p34^{cdc2}$-like protein kinase fraction is of biological significance since phosphorylation of histone H1 is required in

chromosome condensation. Histone H1 is composed of three domains: a central hydrophobic domain that interacts with the core histones in the nucleosome and two basic arms at the amino and carboxyl termini, which are thought to embrace the DNA as it enters and exits the nucleosome. Several sites in these two arms become phosphorylated as cells enter mitosis and these sites are specifically phosphorylated by the $p34^{cdc2}$ protein kinase (Langan et al., 1989). Dividing cells may have up to 50-80 % of their histone H1 molecules phosphorylated, whilst non-dividing cells may have 10 % or less, and the phosphorylation of histone H1 increased about six-fold in late G2 to M phase and then rapidly declines as the nuclei return to G1 phase (Bradbury et al., 1974b). Dephosphorylated histone H1 is used as an effective substrate in *vitro* to detect activity of $p34^{cdc2}$ in late G2 to M phase from yeast to human cells and was effective here as the substrate for activity of $p34^{cdc2}$-like protein kinase from *N. plumbaginifolia*.

A more complete absence of $p34^{cdc2}$-like H1 kinase activity in phases of the cell cycle other than mitosis was observed in cells arrested in G1 phase by nitrogen or phosphorous limitation, and may indicate a more complete synchronisation. Synchronisation in these cultures is nevertheless clearly not complete since both nitrogen-limited and phosphorous-limited cells both subsequently show a proportion of the population that incorporates BrdU into nuclear DNA throughout the sampling period (Fig. 3.9 and 3.12). In nitrogen-synchronised cells an increase in kinase activity began from 21h (Fig. 3.11.b) and nuclear DNA content confirms that cells at 24h are still in G2 phase entering mitosis. These cells maintain activity for a longer period than in the other synchronised cultures (Fig. 3.11) and this may indicate that essential mitosis proteins have been depleted by the nitrogen starvation and only slowly restored. The nuclear DNA content (Fig. 3.9.c) confirms that cells are unable to leave G2 phase by completing mitosis until after 30h. A possible candidate for a protein at a suboptimal concentration and for a normal rate of progress through mitosis is $p34^{cdc2}$-like protein kinase itself, since the maximum activity of this enzyme in cells that are recovering from nitrogen starvation is only 4.5 pmole P per gram per min, compared with 60, 100 and 14 respectively in cells recovering from aphidicolin inhibition, phosphorous or sucrose starvation. In phosphorous-synchronised cells an increase in kinase also begins from 21h

(Fig. 3.14.b). Cells at this time are presumably in G2 phase since at 15h they have clearly increased their nuclear DNA content above G1 levels and are approaching G2 levels (Fig. 3.12.b). This DNA content implies that at 15h they are still in S phase and incorporation of BrdU show that nuclear DNA synthesis is approaching completion at that time (Fig. 3.12.d). In both of the cultures synchronised to G1 phase by nutrient limitation the activity of $p34^{cdc2}$-like H1 kinase is at least 7.5 times higher than in samples containing predominantly interphase cells. It is likely that in individual cells the differential activity between interphase and mitosis is even greater because of the asynchrony present in the cultures that were sampled.

Sucrose limited cells showed some preferential arrest in G2 phase and therefore were expected to resume cycling by entering mitosis, perhaps being suitable for studying activity changes associated with initiation of mitosis. Unfortunately arrest was not uniformly in G2 phase and synchrony was reduced by presence of a significant proportion of G1 cells, as indicated by the DNA profile (Fig. 3.15.a) and by the high background of BrdU incorporation (Fig. 3.15.d). Synchrony was also reduced by the slow resumption of mitotic activity presumably due to the stress of starvation for both carbon and energy. A prolonged duration of $p34^{cdc2}$ kinase activity was observed with a peak at 18-21h. The resulting daughters were detected in their next cell cycle as entering S phase at 27-30h when a peak of BrdU incorporation was detected (Fig. 3.15.d). The sucrose limited cells do confirm the general correlation of $p34^{cdc2}$-like H1 kinase activity with G2 phase.

Clear genetic evidence implicates the cdc2 gene product as necessary for progress from G1 to S phase in budding yeast (Reed, 1980) and fission yeast (Nurse and Bissett, 1981). In budding yeast activity attributed to the *cdc2/CDC28* gene product has been detected in extracts from late G1 cells. There is therefore reason to suspect that catalytic activity of $p34^{cdc2}$-like kinase may be present in the late-G1/S phase of plant cells. The substrate of the kinase at late G1/S is not known in any cell type, although a protein identified only by its 45 kDa size has been implicated in budding yeast (Mendenhall et al., 1987) and during S phase proteins involved in DNA replication are likely candidates (Blow and Nurse, 1990).

Although the natural substrate(s) of cdc2 at late G1 are not available for use as substrates, the entirely heterologous milk protein α-casein, has been used previously as a substrate for the $p34^{cdc2}$ of *S. pombe*. In extracts from that yeast, activity was detected in a fraction precipitated by antibody raised against the carboxyl terminal of $p34^{cdc2}$ that is specific to the kinase. Specificity is also expected because $p34^{cdc2}$ is the only calcium-independent H1 histone kinase present in fission yeast, as indicated by the complete elimination of all such kinase activity at restrictive temperature in extracts of cells containing a single mutation in the *cdc2* gene. Presence of casein kinase activity in G1 phase immunopreciptates of $p34^{cdc2}$ (Simanis and Nurse, 1986) therefore indicates that casein may be a suitable substrate to detect activity of the late G1 form of $p34^{cdc2}$. Casein certainly cannot be used to preferentially detect G1/S activity of $p34^{cdc2}$ since it is clearly phosphorylated by the mitotic form of the cdc2 kinase from yeast (Simanis and Nurse, 1986) and human cells (Draetta and Beach, 1988).

In view of the incomplete synchrony obtained with the suspension cultures studied here, evidence of possible G1/S active forms of $p34^{cdc2}$-like protein kinase must be interpreted with caution. However, an indication of such activity was obtained in the cells synchronised by nitrogen limitation. This activity during the first 9h of culture was at three times the basal level seen during the rest of the cell cycle and it correlated with the period in which incorporation of BrdU into DNA occurred with the highest incidence (Fig. 3.9.d). A weaker casein kinase activity was detected in cells that had been synchronised by phosphate limitation (Fig. 3.14.a). It did not as clearly correlate with DNA replication but in these cells DNA replication was spread over a longer interval, perhaps because of the reduced availability of phosphorylated DNA precursors. Cells previously synchronised by sucrose limitation show low casein kinase activity, which in the phosphorImagere appear more significant in the first 9h (Fig. 3.17.a). This activity may come from cells arrested in G1, which were also detected as the 25% of cells incorporating BrdU (Fig. 3.15.d) but the majority of cells are in G2 phase (Fig. 3.15) and therefore not expected to present G1/S kinase activity. This raises the possibility that an unknown kinase that may be involved in recovery from starvation and therefore present in both nitrogen and sucrose starved cell extracts is present as a contaminant in the

$p13^{suc1}$-bound kinase fraction. However the higher activity and temporal correlation with late G1 and S phase of casein kinase, in cells that were synchronised by nitrogen limitation and were more uniformly in G1/S phases, suggests that part of this activity may be due to $p34^{cdc2}$-like kinase activity involved in the cell cycle. This is the first indication of such activity from a plant cell. Comparison with mammalian cells and the temporal correlation with S phase raise the possibility that this could be in part due to the CDK2 variant of cdc2 (Fang and Newport, 1991) that complexes with cyclin E at late G1 and with cyclin A during S phase (Fang and Newport, 1991) and is involved in driving progress through S phase. It will be interesting to test this hypothesis as specific reagents become available for equivalent proteins in plant cells.

Fig. 3.1. Effects of different concentrations of aphidicolin on the DNA synthesis of suspension culture of *N. plumbaginifolia*. Frequency of occurrence of DNA content observed with propidium iodide staining, in: (a) nuclei of proliferating cells of suspension culture which grew for 48 hour after normal dilution in CSV medium; (b) nuclei of proliferating cells in which synthesis of DNA was blocked with 5 μg/ml of aphidicolin for 24 hour; (c, d) nuclei of cells prevented from synthesising DNA by 24 hour exposure to 10 μg/ml or 20 μg/ml aphidicolin respectively. Aphidicolin was added from a stock solution of 5 mg/ml in DMSO and the resulting maximum concentration of DMSO was 0.4%.

Fig. 3.1

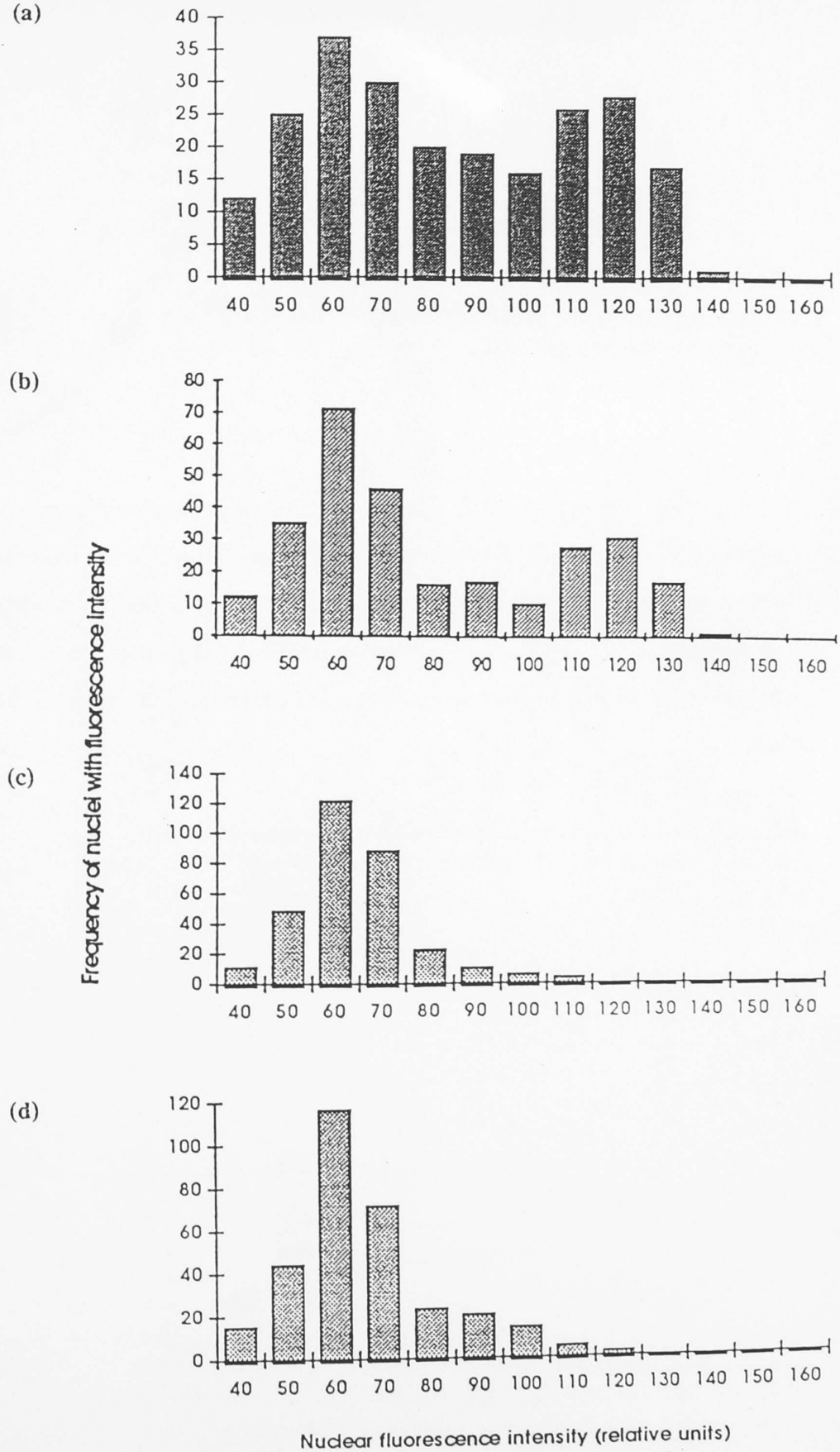
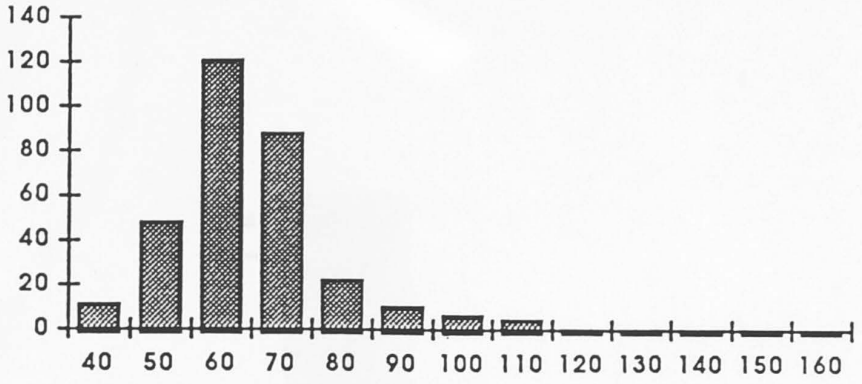

(a)
(b)
(c)
(d)
Frequency of nuclei with fluorescence intensity
Nuclear fluorescence intensity (relative units)
40 50 60 70 80 90 100 110 120 130 140 150 160

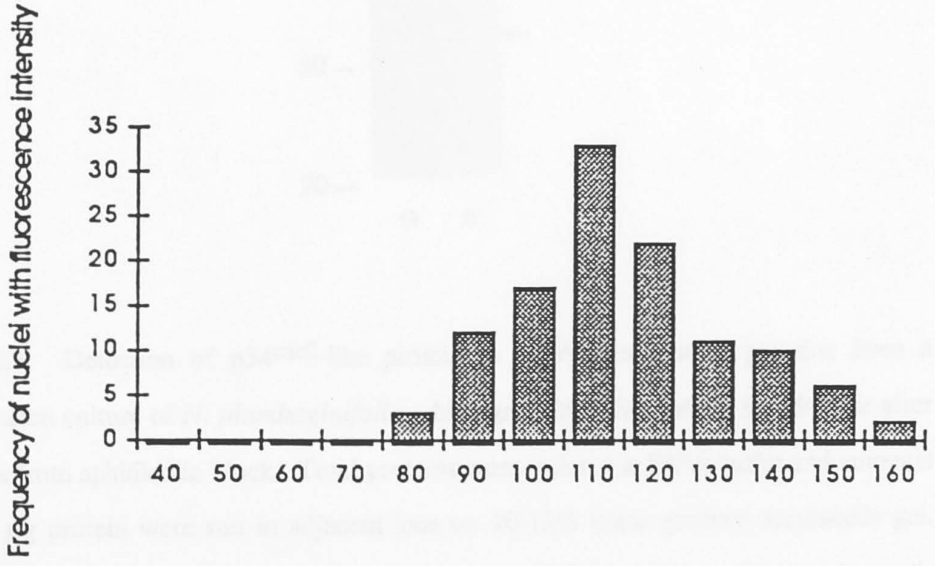
Fig. 3.2. The recovery of DNA synthesis in cells of a suspension culture of *N. plumbaginifolia* after release from the aphidicolin block. Frequency of occurrence of DNA content observed with propidium iodide staining, in: (a) nuclei of cells prevented from synthesising DNA by exposure to 20 μg/ml aphidicolin for 24 hour; (b, c) nuclei of cells grown for 16 and 24 hour respectively after release from 20 μg/ml aphidicolin block.

Fig. 3.2

(a)


140
120
100
80
60
40
20
0
40 50 60 70 80 90 100 110 120 130 140 150 160

(b)


Frequency of nuclei with fluorescence intensity
35
30
25
20
15
10
5
0
40 50 60 70 80 90 100 110 120 130 140 150 160

(c)

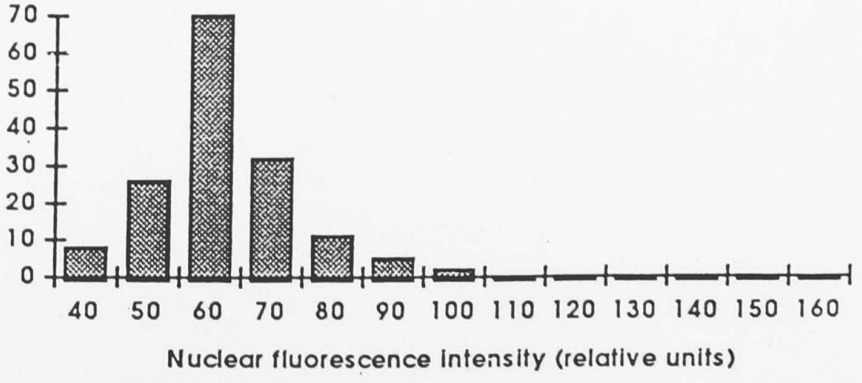

70
60
50
40
30
20
10
0
40 50 60 70 80 90 100 110 120 130 140 150 160
Nuclear fluorescence intensity (relative units)

Fig. 3.3

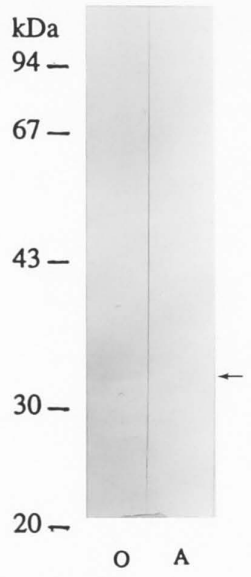


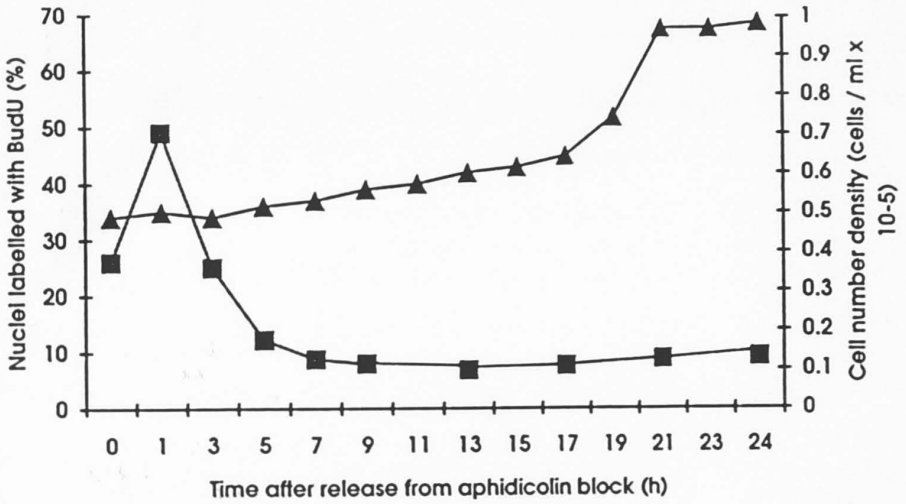
Fig. 3.3. Detection of $p34^{cdc2}$-like protein in a Western blot of proteins from a suspension culture of *N. plumbaginifolia* which grew in CSV medium for 19 hour after release from aphidicolin block. Total proteins were isolated in RIPA buffer and amounts of 50 µg protein were run in adjacent lane on 10-15% linear gradient acrylamide gel. Transferred proteins were probed with affinity-purified polyclonal anti-EGVPSTAIREISLLKE antibody. Antibody was divided and preincubated, (0) without addition, (A) with 20 nM EGV peptide EGVPSTAIREISLLKE.

Fig. 3.4. The effects of release of aphidicolin inhibition on cell number density, DNA synthesis and level of $p34^{cdc2}$-like protein in a suspension culture of *N. plumbaginifolia*. Cells were transferred into fresh CSV medium after blocking with 20 μg/ml aphidicolin for 24h. (a) shows the change of cell number density (▲), and the incidence of nuclei labelled with BrdU during (a) (■).

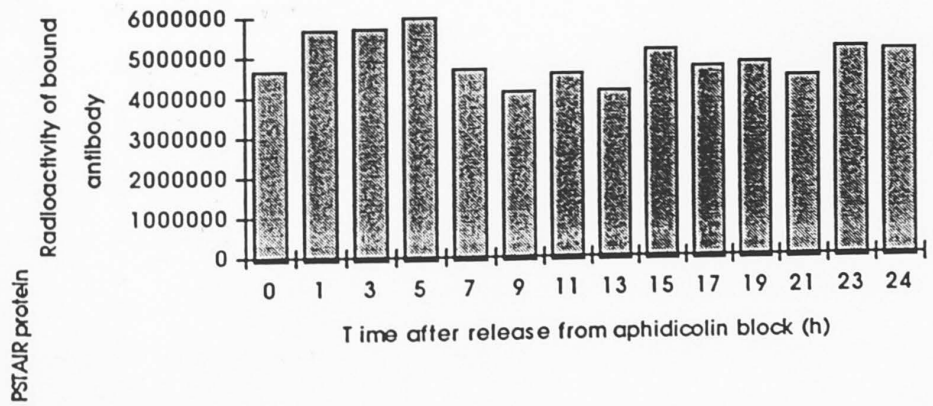
(b) shows the change in level of $p34^{cdc2}$-like protein (PSTAIR protein) in the suspension culture synchronised by aphidicolin as shown in (a). Equal loadings of 50 μg protein were separated on a 10-15% linear gradient acrylamide gel. Transferred proteins were probed with affinity-purified polyclonal anti-EGVPSTAIREISLLKE antibody and bound antibody was detected by ^{125}I anti-rabbit IgG. The image shown in (c) was obtained by exposure in a phosphorImager and analysed to determine levels of $p34^{cdc2}$-like protein by quantification of bound isotope (b).

Fig. 3.4

(a)


Nuclei labelled with BudU (%)
Cell number density (cells / ml x 10-5)
Time after release from aphidicolin block (h)

(b)


PSTAIR protein
Radioactivity of bound antibody
T ime after release from aphidicolin block (h)

(c)

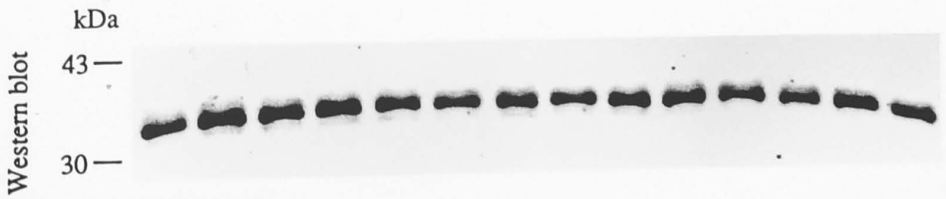

kDa
Western blot
43
30

Fig. 3.5. (a) shows the activity of $p34^{cdc2}$-like protein kinase in cells synchronised by aphidicolin and sampled as shown in Fig. 3.4.a. $p34^{cdc2}$-like protein kinase in 0.1g of fresh weight of cells was purified by 20 µl of $p13^{suc1}$ beads and after washing was eluted with 50 µl of 0.5 mg/ml of free $p13^{suc1}$. The activity of $p34^{cdc2}$-like protein kinase was measured, using histone H1 as a substrate, at 30°C for 10 min. The amount of ^{32}P transferred was measured by placing 20 µl of reaction mixture on P81 phosphocellulose paper and counting in a scintillation counter. PhosphorImaged histone H1 was obtained by loading 30 µl of reaction mixture on a 12% acrylamide gel and exposure in a phosphorImager.

(b) shows the linearity of recovery of $p34^{cdc2}$-like kinase from a suspension culture of *N. plumbaginifolia*. Cells were cultivated in fresh CSV for 19h after release from the aphidicolin block as shown in (a). The $p34^{cdc2}$-like protein kinase in 0.016, 0.032, 0.064, 0.096, 0.128, 0.192g of cells was purified respectively by 40 µl of $p13^{suc1}$ beads and eluted with 50 µl of 0.5 mg/ml of free $p13^{suc1}$. The activity of $p34^{cdc2}$-like protein kinase was measured, using histone H1 as a substrate, at 30°C for 10 min. The amount of ^{32}P transferred was measured by placing 20 µl of reaction mixture on P81 phosphocellulose paper and counting in a scintillation counter. PhosphorImaged histone H1 shown was obtained by loading 30 µl of reaction mixture on a 12% acrylamide gel and exposure in a phosphorImager.

Fig. 3.5

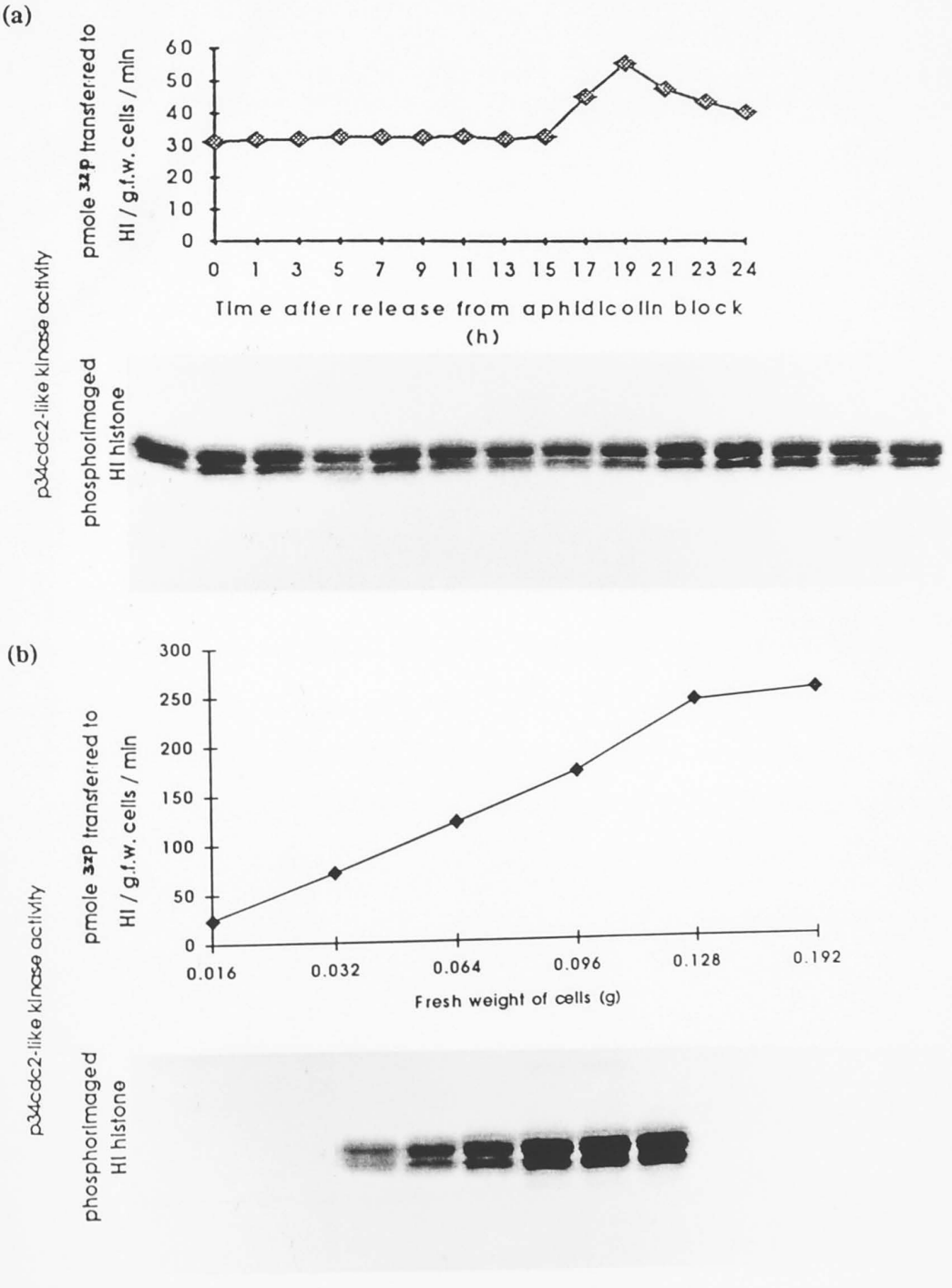
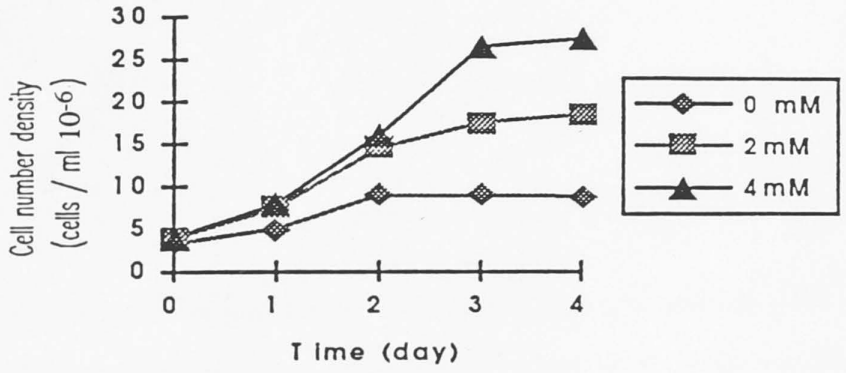

(a)
p34cdc2-like kinase activity
pmole 32P transferred to HI / g.f.w. cells / min
60
50
40
30
20
10
0
0 1 3 5 7 9 11 13 15 17 19 21 23 24
Time after release from aphidicolin block (h)
phosphorimaged HI histone
(b)
p34cdc2-like kinase activity
pmole 32P transferred to HI / g.f.w. cells / min
300
250
200
150
100
50
0
0.016
0.032
0.064
0.096
0.128
0.192
Fresh weight of cells (g)
phosphorimaged HI histone

Fig. 3.6. Effects of limiting concentrations of nitrogen in CSV medium on cell division and nuclear DNA content of a suspension culture of *N. plumbaginifolia*. Cells were washed twice by centrifugation in nitrogen free CSV medium after growth in complete CSV medium for 4 days and were spun down and then resuspended in different concentrations of nitrogen media at the same cell number density. Cell number density was determined by staining nuclei with DAPI. Nuclear DNA content was measured by quantifying fluorescence intensity of nuclei stained with propidium iodide. (a): cell number density of cells in 0, 2 and 4 mM nitrogen in CSV medium; (b): distribution of frequency of nuclear fluorescence intensities in cells cultivated in 0 mM nitrogen CSV medium for 4 days.

Fig. 3.6

(a)


Cell number density
(cells / ml 10-6)
30
25
20
15
10
5
0
0
1
2
3
4
Time (day)
0 mM
2 mM
4 mM

(b)

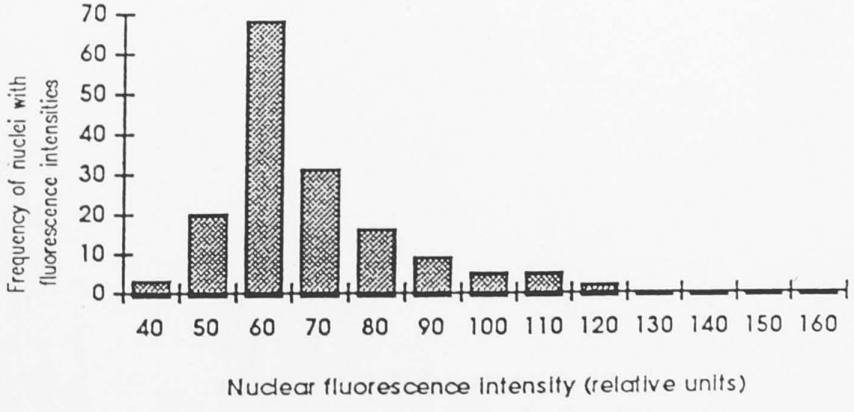
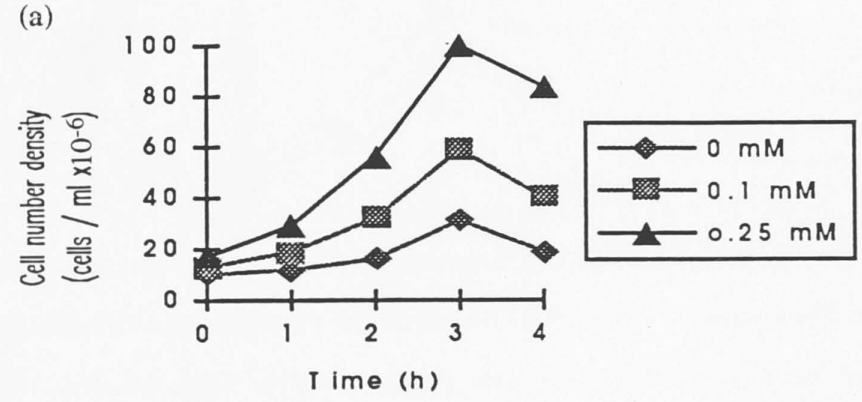

Frequency of nuclei with fluorescence intensities
70
60
50
40
30
20
10
0
40
50
60
70
80
90
100
110
120
130
140
150
160
Nuclear fluorescence intensity (relative units)

Fig. 3.7. The effect of different concentrations of phosphate on cell proliferation and on nuclear DNA content of cells limited by phosphate, shown here, was studied in parallel with the effect of limiting nitrogen (Fig. 3.6) and limiting sucrose (Fig. 3.8). Cells were washed twice by centrifugation in phosphate free CSV medium after growth in complete CSV medium for 4 days and were spun down and then resuspended in different concentrations of phosphate media at the same cell number density. (a) shows the cell number density of cells in 0, 0.1, 0.25 mM phosphate in CSV medium; (b) shows the distribution of nuclear fluorescence intensities in cells cultivated in 0 mM phosphate CSV medium for 4 days.

Fig. 3.7


(a)
Cell number density
(cells / ml x10-6)
100
80
60
40
20
0
0
1
2
3
4
Time (h)
0 mM
0.1 mM
o.25 mM

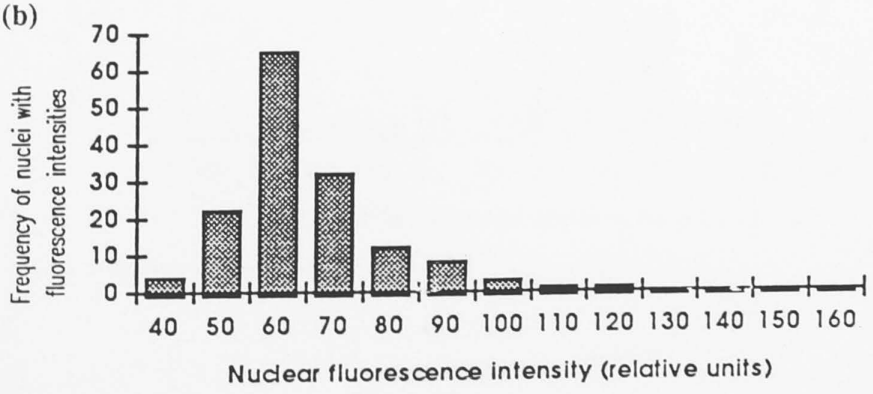
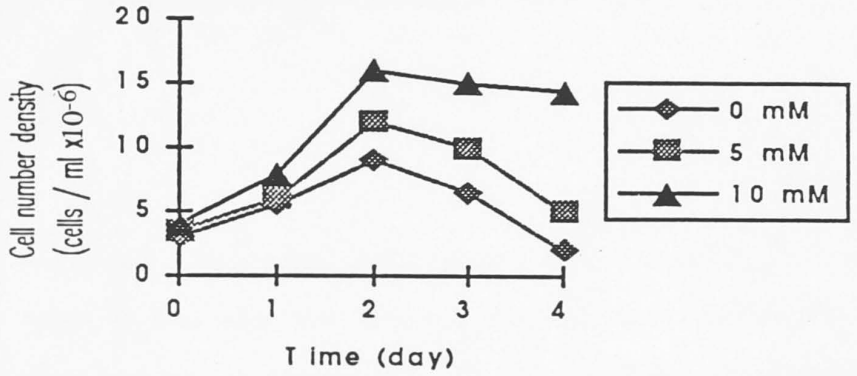

(b)
Frequency of nuclei with
fluorescence intensities
70
60
50
40
30
20
10
0
40 50 60 70 80 90 100 110 120 130 140 150 160
Nuclear fluorescence intensity (relative units)

Fig. 3.8. The effect of different concentrations of sucrose on cell proliferation and on nuclear DNA content of cells limited by sucrose, shown here, was studied in parallel with studying the effect of limiting nitrogen and phosphorous (Figs. 3.6, 3.7) . Cells were washed twice by centrifugation in sucrose free CSV medium and were spun down and then resuspended in different concentrations of sucrose media at the same cell number density. (a) shows cell number density of cells in 0, 5, 10 mM sucrose in CSV medium; (b) shows the distribution of frequency of nuclear fluorescence intensities in cells cultivated in 0 mM sucrose in CSV medium for 4 days.

Fig. 3.8

(a)


20
15
10
5
0
Cell number density
(cells / ml x10-6)
0
1
2
3
4
T ime (day)
0 mM
5 mM
10 mM

(b)

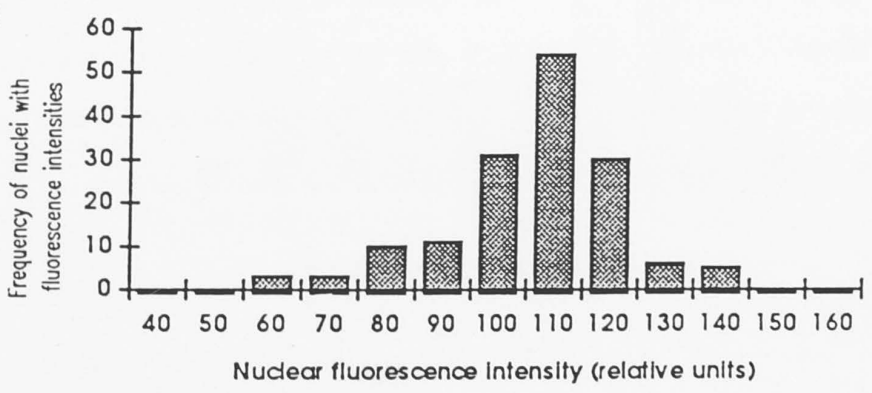

60
50
40
30
20
10
0
Frequency of nuclei with
fluorescence intensities
40 50 60 70 80 90 100 110 120 130 140 150 160
Nuclear fluorescence intensity (relative units)

Fig. 3.9. Change of the nuclear DNA content in a suspension culture of *N. plumbaginifolia* synchronised by nitrogen starvation then provided with complete medium during the sampling period. Cells were cultivated in 0 mM nitrogen in CSV medium for 4 days as shown in Fig. 3.6 and then spun down and resuspended in complete CSV medium and sampled in the subsequent 33h. Nuclear DNA content was estimated by measuring nuclear fluorescence intensity of nuclei stained with propidium iodide. (a), (b) and (c) show distribution of nuclear fluorescence intensities in cells cultivated in complete CSV medium for 0h, 24h, 30h respectively after release from nitrogen starvation. (d) shows the incidence of cells synthesising nuclear DNA when cultivated in complete CSV medium after release from nitrogen starvation detected by incubating samples of culture in 100 μM BrdU for 30 min then probing for presence of incorporated base analogue in nuclear DNA

Fig. 3.9

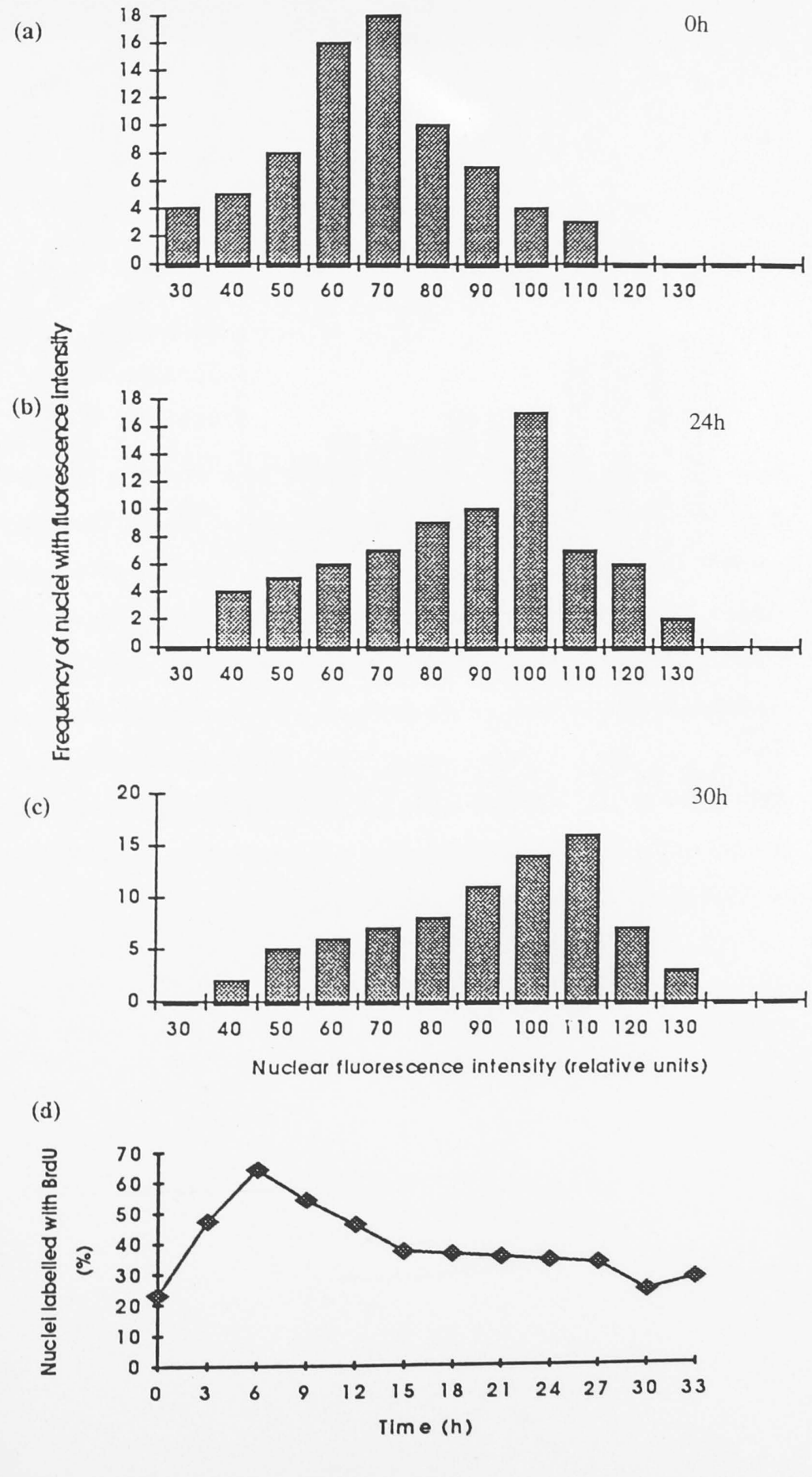

(a)
0h
(b)
24h
(c)
30h
Frequency of nuclei with fluorescence intensity
Nuclear fluorescence intensity (relative units)
(d)
Nuclei labelled with BrdU (%)
Time (h)

Fig. 3.10. Changes in the level of $p34^{cdc2}$-like protein relative to other protein in suspension cultures of *N. plumbaginifolia* after release of nitrogen starvation. After growth in zero nitrogen medium for 4 days cells were spun down and resuspended in complete CSV medium and sampled in subsequent 33h. Total proteins were extracted from 0.1g of frozen cell powder in RIPA buffer. Equal loadings of 50 μg protein were separated on a 10-15% linear gradient acrylamide gel. Transferred proteins were probed with affinity-purified polyclonal anti-EGVPSTAIREISLLKE antibody and bound antibody was detected by ^{125}I anti-rabbit IgG. The image shown in (b) was obtained by exposure in a phosphorImager and analysed to determine levels of $p34^{cdc2}$-like protein by quantification of bound isotope as shown in (a).

Fig. 3.10

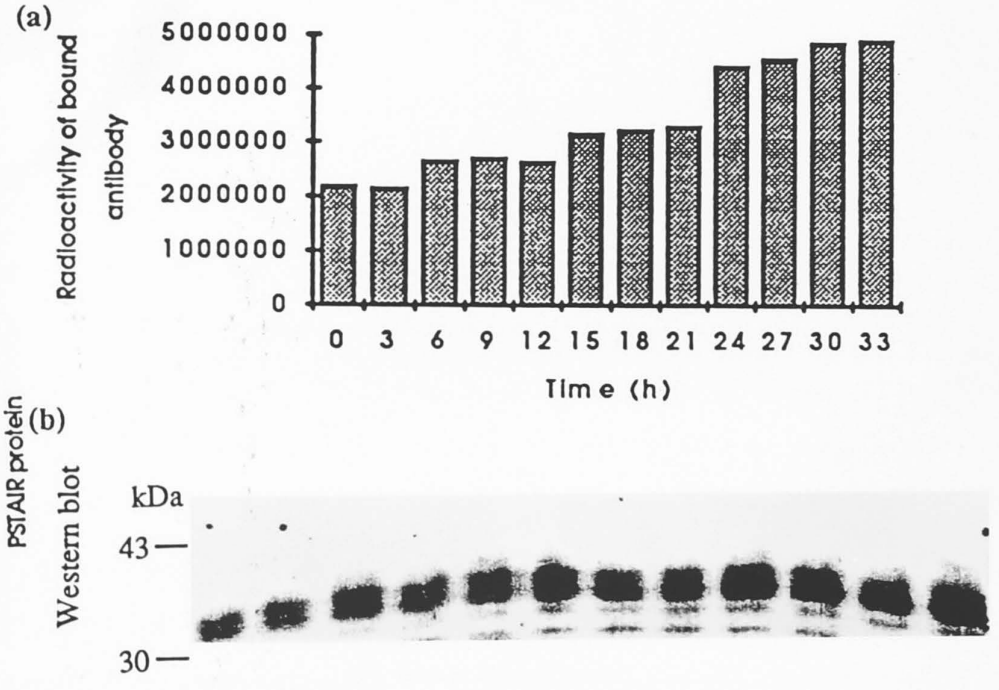
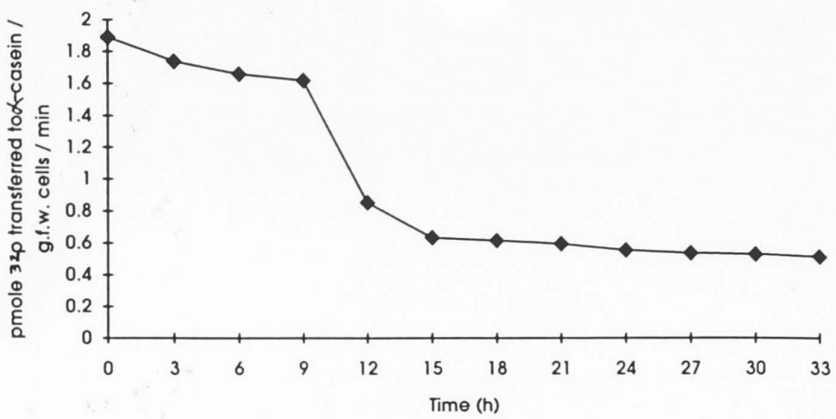

(a)
Radioactivity of bound antibody
5000000
4000000
3000000
2000000
1000000
0
0 3 6 9 12 15 18 21 24 27 30 33
Time (h)
(b)
PSTAIR protein
Western blot
kDa
43
30

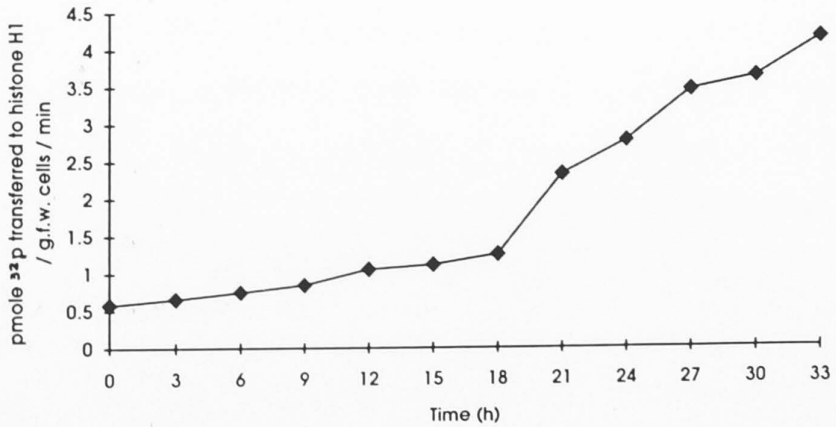
Fig. 3.11. Samples assyed for $p34^{cdc2}$-like protein after synchronisation by nitrogen starvation (Fig. 3.9) were also assayed for $p34^{cdc2}$-like protein kinase. The enzyme was purified from 0.1g of frozen cell powder in NDE buffer by 20 µl of $p13^{suc1}$ beads and eluted with 50 µl of 0.5 mg/ml of $p13^{suc1}$ solution. The activity of $p34^{cdc2}$-like protein kinase was measured using histone H1 as a substrate shown in (b) and α-casein as substrate shown in (a) at 30°C for 10 min. The amount of ^{32}P transferred was measured by placing 20 µl of reaction mixture on P81 phosphocellulose paper and counting in a scintillation counter. PhosphorImaged histone H1 or α-casein shown was obtained by loading 30 µl of reaction mixture on a 12% acrylamide gel and exposure in a phosphorImager.

Fig. 3.11

(a)



(b) p34cdc2-like kinase activity



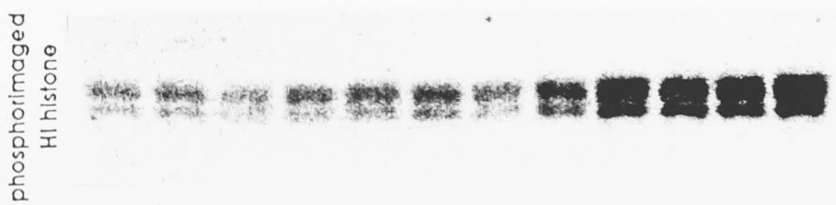


Fig. 3.12. Change in frequency of nuclear DNA content and of cells synthesising nuclear DNA in suspension cultures of *N. plumbaginifolia* after release of phosphate starvation. After growth in 0 mM phosphate for 4 days as shown in Fig. 3.7 cells were spun down, resuspended in complete CSV medium and sampled in the subsequent 33h. Nuclear DNA content was estimated by measuring fluorescence intensity of nuclei stained with propidium iodide. (a), (b) and (c) respectively show the frequency distribution of nuclear fluorescence intensities in cells that were cultivated in complete CSV medium for 0h, 15h and 30h respectively after release from phosphate starvation. (d) shows the incidence of cells synthesising nuclear DNA when cultivated in complete CSV medium from 0h to 33h after release from phosphate starvation. DNA synthesis was detected by incubating samples of culture with 100 μM BrdU for 30 min then probing for presence of incorporated base analogue in nuclear DNA.

Fig.3.12

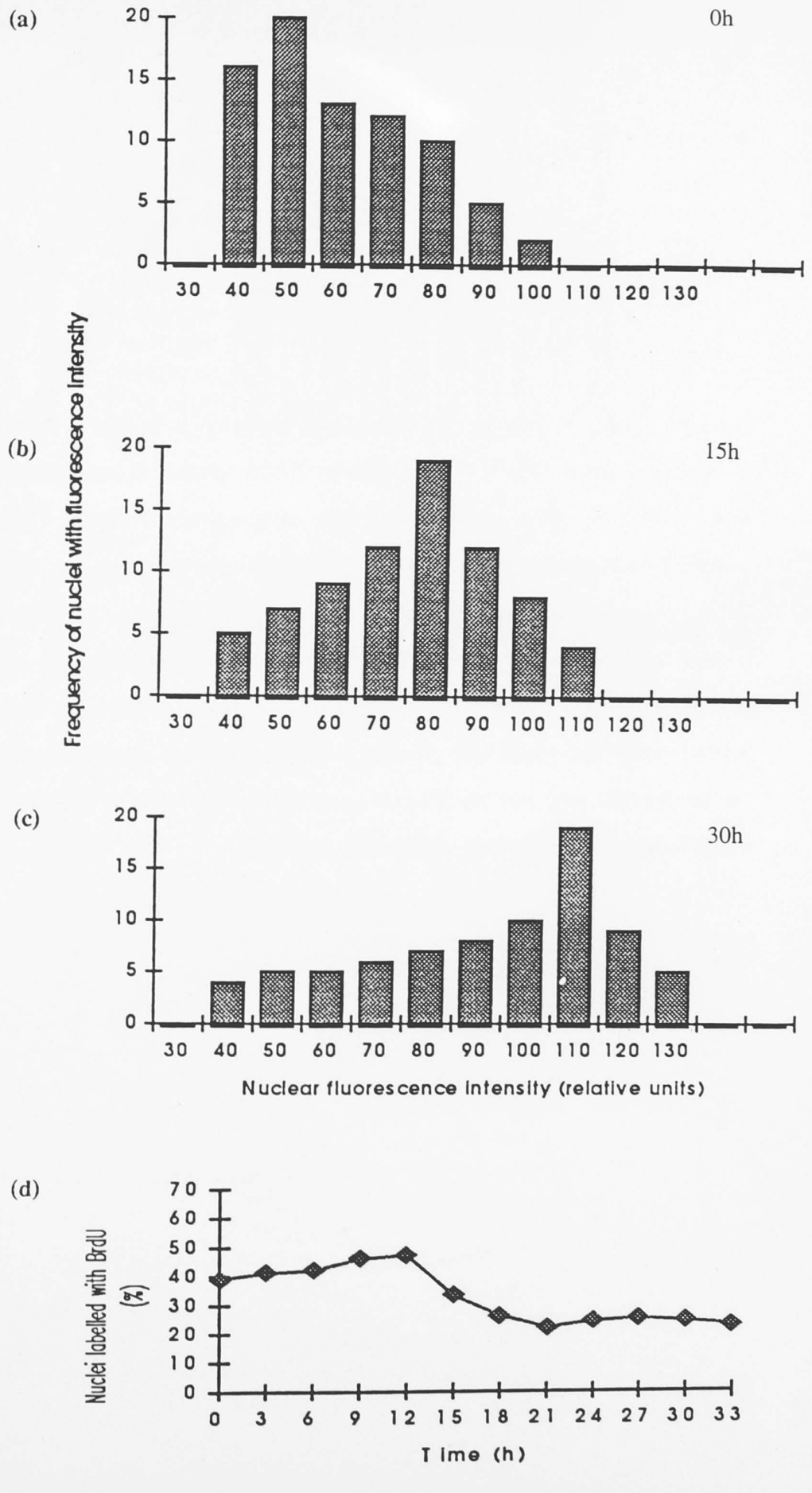

(a)
0h
20
15
10
5
0
30 40 50 60 70 80 90 100 110 120 130
Frequency of nuclei with fluorescence intensity
(b)
15h
20
15
10
5
0
30 40 50 60 70 80 90 100 110 120 130
(c)
30h
20
15
10
5
0
30 40 50 60 70 80 90 100 110 120 130
Nuclear fluorescence intensity (relative units)
(d)
Nuclei labelled with BrdU (%)
70
60
50
40
30
20
10
0
0 3 6 9 12 15 18 21 24 27 30 33
Time (h)

Fig. 3.13. Cells resuming after phosphate starvation, shown in Fig.12, further analysed to determine levels of $p34^{cdc2}$-like protein. After growth in zero phosphate CSV medium for 4 days (Fig. 3.7) cells were spun down and resuspended in complete CSV medium and sampled in subsequent 33h. Total proteins were extracted from 0.1g of frozen cell powder in RIPA buffer. Equal loadings of 50 μg protein were separated on a 10-15% linear gradient acrylamide gel. Transferred proteins were probed with affinity-purified polyclonal anti-EGVPSTAIREISLLKE antibody and bound antibody was detected by ^{125}I anti-rabbit IgG. The image shown in (b) was obtained by exposure in a phosphorImager and was analysed to determine levels of $p34^{cdc2}$-like protein by quantification of bound isotope as shown in (a).

Fig. 3.13

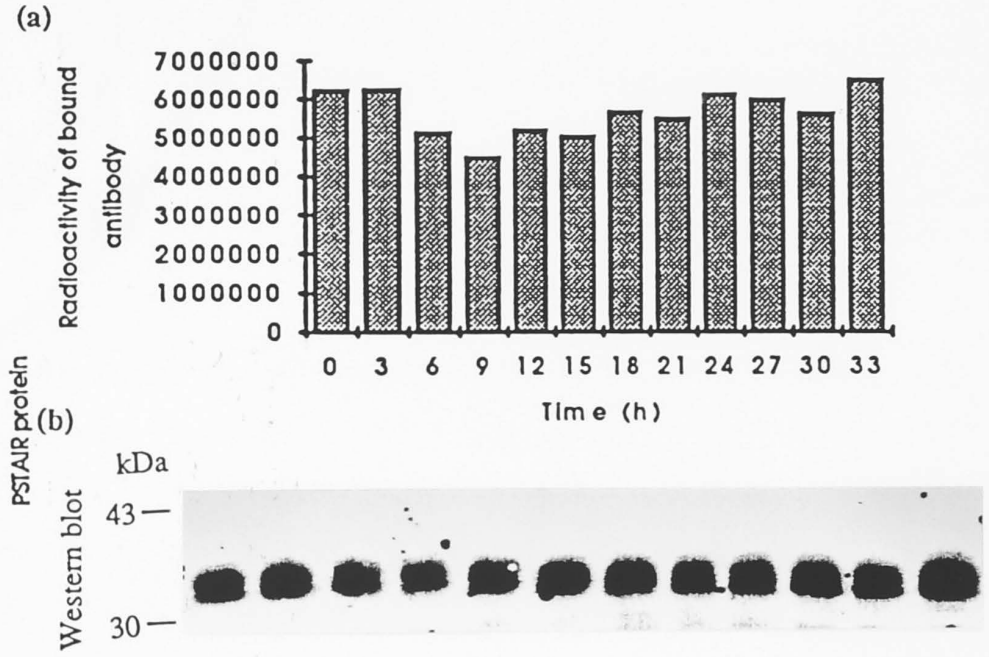
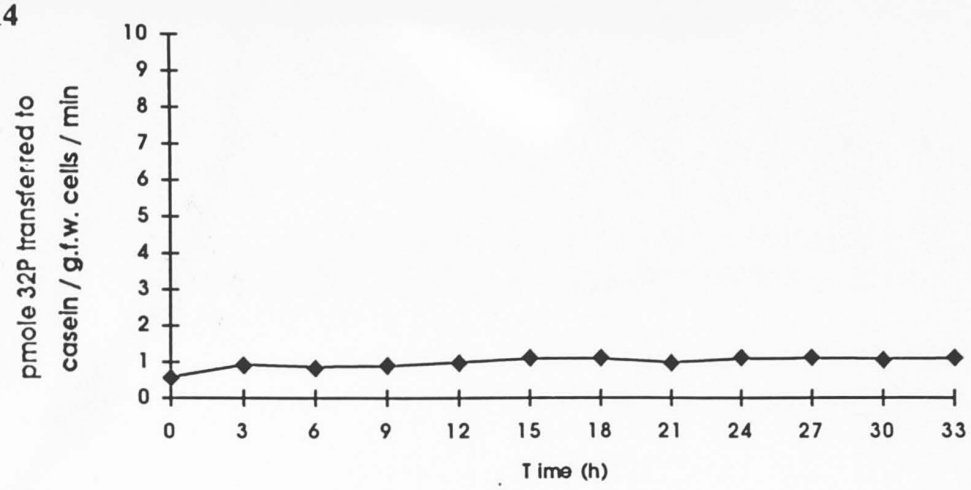

(a)
Radioactivity of bound antibody
7000000
6000000
5000000
4000000
3000000
2000000
1000000
0
0 3 6 9 12 15 18 21 24 27 30 33
Time (h)
(b)
PSTAIR protein
Western blot
kDa
43
30

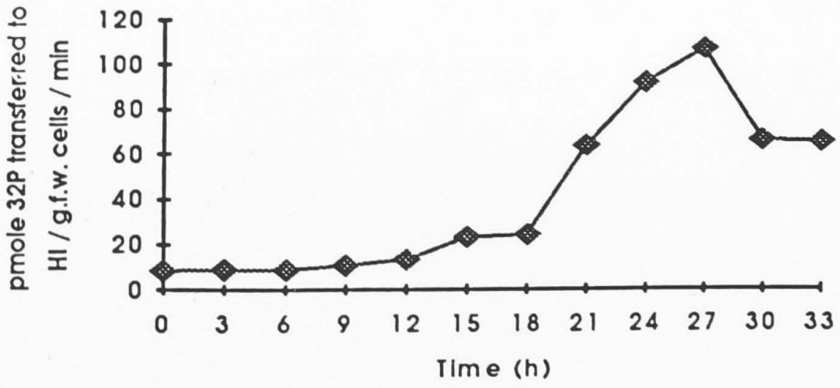
Fig. 3.14. Cells resuming after phosphate starvation, shown in Fig. 3.12 and 3.13 were further analysed to determine the change of activity of $p34^{cdc2}$-like protein kinase. Cells were transferred into complete CSV medium after growth in zero phosphate CSV medium for 4 days and sampled in the subsequent 33h. $p34^{cdc2}$-like protein kinase was purified from 0.1g of frozen cell powder in NDE buffer by 20 µl of $p13^{suc1}$ beads and eluted with 50 µl of 0.5 mg/ml of $p13^{suc1}$ solution. The activity of $p34^{cdc2}$-like protein kinase was measured using histone H1 as a substrate as shown in (a) and using α-casein as a substrate as shown in (b). The amount of ^{32}P transferred was measured by placing 20 µl of reaction mixture on P81 phosphocellulose paper and counting in a scintillation counter. PhosphorImaged histone H1 or α-casein shown was obtained by loading 30 µl of reaction mixture on a 12% acrylamide gel and exposure in a phosphorImager.

Fig. 3.14

(a)



(b) p34cdc2-like kinase activity



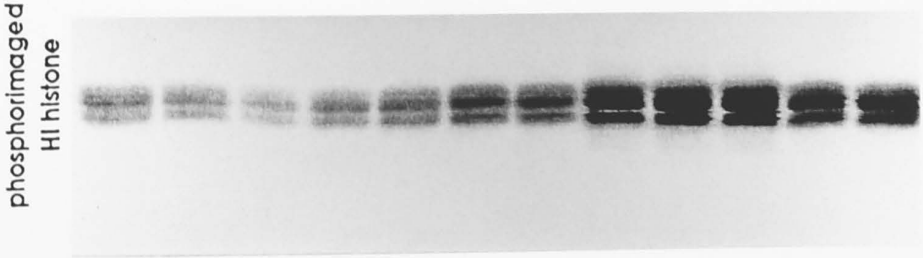


Fig. 3.15. Change in frequency of nuclear DNA content and of cells synthesising nuclear DNA in suspension cultures of *N. plumbaginifolia* after release from sucrose starvation. After growth in 0 mM sucrose for 4 days (Fig. 3.8) cells were spun down and resuspended in complete CSV medium and sampled in the subsequent 33h. Nuclear DNA content was estimated by measuring fluorescence intensity of nuclei stained with propidium iodide. (a), (b) and (c) show respectively the frequency of nuclear fluorescence intensities in cells cultivated in complete CSV medium for 0h, 15h and 24 h after release from sucrose starvation. (d) shows the incidence of cells synthesising nuclear DNA cultivated in complete CSV medium from 0h to 33h after release from sucrose starvation. DNA synthesis was detected by incubating samples of culture with 100 μM BrdU for 30 min then fixing the cells and probing for presence of the base analogue incorporated into DNA.

Fig. 3.15

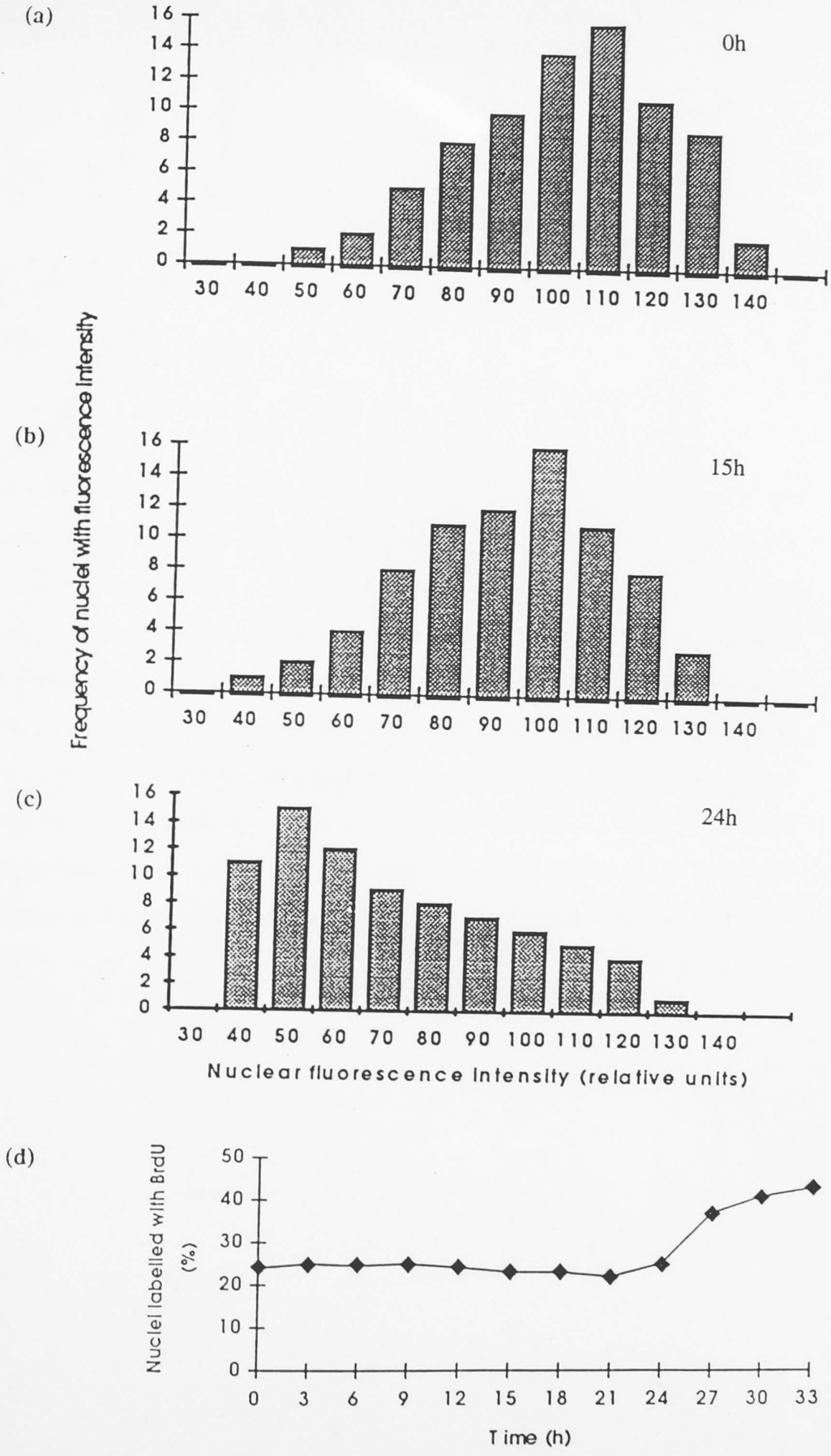

(a)
0h
(b)
15h
(c)
24h
Frequency of nuclei with fluorescence intensity
16
14
12
10
8
6
4
2
0
30 40 50 60 70 80 90 100 110 120 130 140
Nuclear fluorescence intensity (relative units)
(d)
Nuclei labelled with BrdU (%)
50
40
30
20
10
0
0 3 6 9 12 15 18 21 24 27 30 33
Time (h)

Fig. 3.16. Samples shown in Fig. 3.15 were also analysed to determine the levels of $p34^{cdc2}$-like protein in suspension cultures of *N. plumbaginifolia* that were synchronised by sucrose starvation and resumed proliferation during the sampling period. After growth in zero sucrose CSV medium for 4 days cells were cultivated in complete CSV medium for 33h. Total proteins were extracted from 0.1g of frozen cell powder in RIPA buffer. Equal loadings of 50 μg protein were separated on a 10-15% linear gradient acrylamide gel. Transferred proteins were probed with affinity-purified polyclonal anti-EGVPSTAIREISLLKE antibody and bound antibody was detected by ^{125}I anti-rabbit IgG. The image shown in (b) was obtained by exposure in a phosphorImager and analysed to determine levels of $p34^{cdc2}$-like protein by quantification of bound isotope as shown in (a).

Fig. 3.16

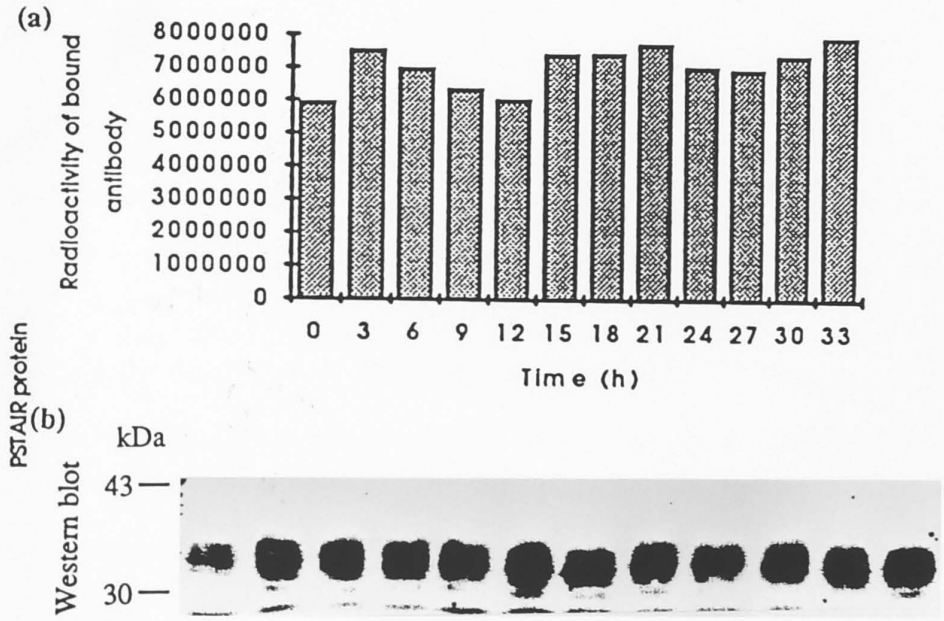
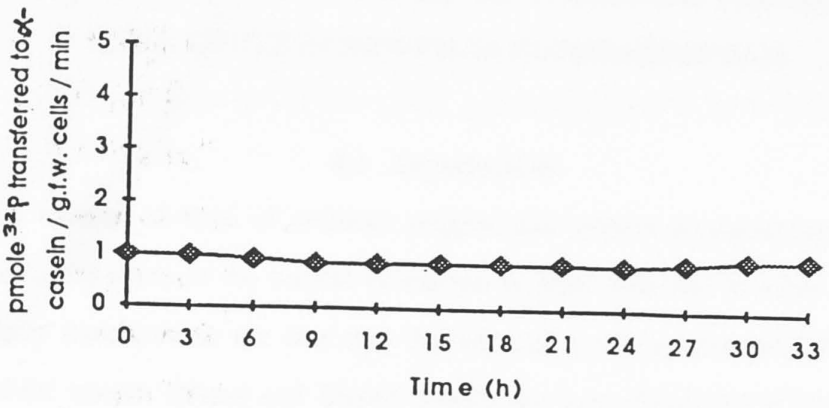

(a)
Radioactivity of bound antibody
8000000
7000000
6000000
5000000
4000000
3000000
2000000
1000000
0
0 3 6 9 12 15 18 21 24 27 30 33
Time (h)
PSTAIR protein
(b)
Western blot
kDa
43
30

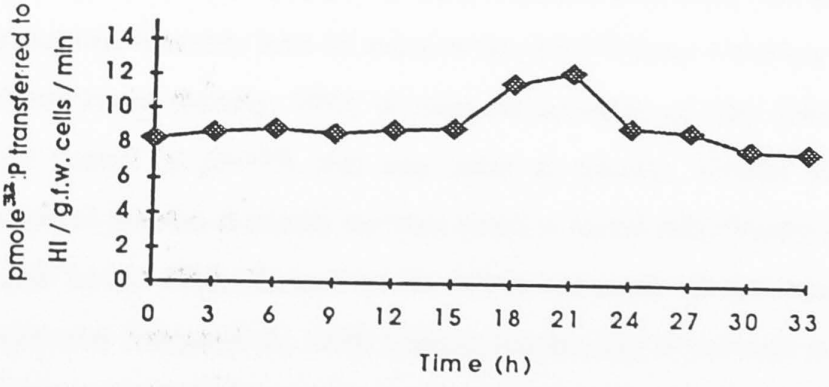
Fig. 3.17. Samples shown in Figs 3.15 and 3.16 were also analysed to determine the activity of $p34^{cdc2}$-like protein kinase in *N. plumbaginifolia* cells synchronised by previding sucrose starvation. After growth in zero sucrose CSV medium for 4 days cells were transferred into complete CSV medium and sampled in the subsequent 33h. $p34^{cdc2}$-like protein kinase was purified from 0.1g of cell powder in NDE buffer by 20 µl of $p13^{suc1}$ beads and eluted with 50 µl of 0.5 mg/ml of $p13^{suc1}$ solution. The activity of $p34^{cdc2}$-like protein kinase was measured using histone H1 as a substrate as shown in (b) and α-casein as a substrate as shown in (a) at 30°C for 10 min. The amount of ^{32}P transferred was measured by placing 20 µl of reaction mixture on P81 phosphocellulose paper and counting in a scintillation counter. PhosphorImaged histone H1 or α-casein shown was obtained by loading 30 µl of reaction mixture on a 12% acryl amide gel and exposure in a phosphorImager.

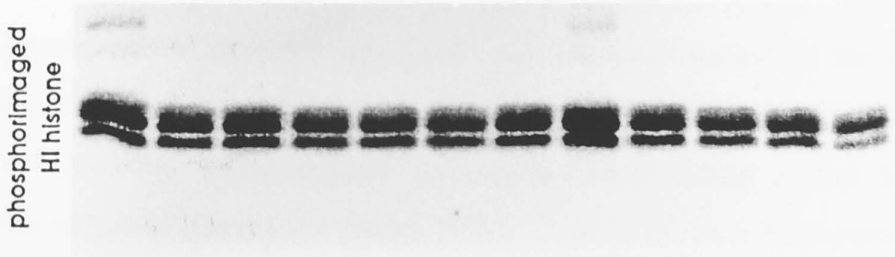
Fig. 3.17

(a)



(b) p34cdc2-like kinase activity





CHAPTER 4

EFFECTS OF OKADAIC ACID, AN INHIBITOR OF TYPE 2A PHOSPHOPROTEIN PHOSPHATASES, ON PROGRESS THROUGH THE CELL CYCLE IN *NICOTIANA PLUMBAGINIFOLIA*

4.1 Introduction

A number of lines of evidence suggest that protein phosphorylation may be particularly important in the control of mitosis in plant cells and in other eukaryotes. Particularly significant is the fact that the key cell cycle protein, $p34^{cdc2}$ which is essential for mitosis (Nurse and Bisselt, 1981), has been conserved in all eukaryotes (Nurse, 1990) and is capable of defining whether the next cell cycle event is DNA replication or mitosis (Broek et al., 1991), is a protein kinase. Protein phosphorylation is important in determining the activity of the $p34^{cdc2}$ kinase, which must be phosphorylated and then partially dephosphorylated to release its catalytic activity at mitosis (Nurse, 1990, as described more fully in the introduction to this thesis). Phosphorylation of many proteins is also quantitatively more abundant at mitosis and this may derive directly from the action of the $p34^{cdc2}$ kinase, which has been termed a workhorse kinase (Murray, 1992) or from the activation of other kinases, perhaps under the control of $p34^{cdc2}$, that may occur at mitosis. Greater abundance of phosphorylated proteins at mitosis has been noted in animal cells (Vandre et al., 1984; Maller and Smith, 1985; Karsenti et al., 1987) and study of the unicellular plant *Chlamydomonas* (Harper et al., 1990) suggests that this may be universal in eukaryotes. Protein phosphorylation at mitosis may be involved in changes of nuclear and cytoskeleton structure. For example, the nuclear envelope lamina, which is a supramolecular protein assembly associated with the inner surface of the nuclear membrane contains three predominant polypeptide components, lamins A, B and C, which are reversibly phosphorylated and depolymerised during nuclear evelope breakdown at mitosis (Gerace and Blobel, 1980). There is evidence from experiments with thermolabile $p34^{cdc2}$ enzyme in live yeast cells that $p34^{cdc2}$ activity is essential for

lamin phosphorylation (Luscher et al., 1991). Phosphorylation may also regulate the activity of microtubule associated protein (Gotoh et al., 1991; Matsuda et al., 1992), and phosphoproteins in mitotic spindles may modify the assembly or disassembly of mitotic structures according to their state of phosphorylation. The thiophosphorylated 205 kd peptide, which is localized in the spindle midzone, may be either a positive regulator or mechanochemical transducer of microtubule sliding when it is in a phosphorylated state (Wordeman and Cande, 1987).

The transience with which many division proteins are phosphorylated indicates that activity of phosphoprotein phosphatase enzymes must be significant. Some of these phosphatases that are involved in division have been recognised by mutation. In *Aspergillus* a mutation that blocks completion of anaphase has identified the gene *bim*G, which has a predicted translation product with 86% sequence identity to mammalian phosphoprotein phosphatase type 1 (Doonan and Morris, 1989). In fission yeast, mutations in *dis/bws* and *sds21* have identified genes that are essential for anaphase and have sequence homology to phosphatase type 1 (Ohkura et al., 1989; Booher and Beach, 1989). The *cdc25* gene, which stimulates mitosis in a dose-dependent way (Russell and Nurse, 1986), is necessary for the activation of the mitotic protein $p34^{cdc2}$ (Gould and Nurse, 1989) and encodes a protein tyrosine phosphatase (Draetta et al., 1988; Moreno and Nurse, 1991).

Okadaic acid, which is a long chain polyether fatty acid synthesised by marine dinoflagellates, is an inhibitor of type 1 and type 2A protein phosphatases (Bialojan and Takai, 1988) that raises the general levels of phosphorylation in animal cells, and affects phosphorylation and activity of the $p34^{cdc2}$ homologue in *Xenopus* oocytes (Haystead et al., 1989; Felix et al., 1990; Lee et al., 1991). Plants have been shown to contain protein phosphatases with the same sensitivities to okadaic acid (MacKintosh and Cohen, 1989). In order to get information concerning the effects of protein phosphorylation and dephosphorylation on the cell division and on the associated cytoskeletal changes in higher plants okadaic acid has been used as an inhibitor to perturb the balance between phosphorylation and dephosphorylation during cell division cycle in cell suspension culture of *N. plumbaginifolia.*

4.2 Materials and Methods

4.2.1. Materials

Two lines of suspension cultured cells of *N. plumbaginifolia* NPT5 were used in this study. A long-term suspension culture (old cell line), which was described in Chapter 3, had been maintained in culture for more than five years and now lacks preprophase bands and the ability to regenerate plants when cells are transferred to solid hormone-containing media (described in Chapter 5). A short-term suspension culture (new cell line) was recently created by myself. This line forms preprophase bands of microtubules and has the ability to regenerate plants when cells are cultured on solid media.

4.2.2. Methods

4.2.2.1. Preparation of cell suspension culture

The newly-extended leaves of *N. plumbaginifolia* were sterilised for half min in 70% ethanol, then 5 min in hypochlorite (12.5 g/l available chlorine; 1/10 dilution of stock), and finally washed three times with sterile water. Squares of tissue (5 mm x 5 mm) were cultured on solid MS medium containing 0.2 mg/l 2,4-D and 0.2 mg/l kinetin and incubated at 26°C with 8h photo period. The resulting callus was transferred to suspension culture. Both old and new cell lines were cultured in 250 ml flasks containing 48 ml of CSV medium with 2 mg/l 2,4-D and 0.05 mg/l kinetin. They were diluted at intervals between 4 and 7 days to 1 x 10^6 cells per ml by mixing 8 ml of suspension with 40 ml of fresh medium.

4.2.2.2. Synchronisation with aphidicolin for study of preprophase band timing was performed as described in Chapter 3.

4.2.2.3. Treatment of cell suspension culture with okadaic acid was performed in freshly diluted cultures of 1,200 µl containing 1.2% (v/v) DMSO, which were incubated with shaking in 55 mm x 25 mm dia flat bottom glass tubes inclined at 45° to the vertical. Control cultures received DMSO without inhibitor.

4.2.2.4. Labelling of phosphoproteins with $[^{32}P]PO_4$.

Labelling of phosphoproteins was performed in freshly diluted 1,200 µl cultures that were supplemented to 3 µCi/ml with carrier-free [^{32}P]PO_4 adjusted to medium pH, and were incubated in parallel in the presence and absence of okadaic acid. Cells were harvested during maximum mitotic activity after 16 hour growth (as seen in Fig. 4.2.a) by centrifugation at 5,000 x g for 5 min and proteins were extracted in RIPA buffer containing protease and phosphatase inhibitors, subject to SDS PAGE on 12% gel and transferred to nitrocellulose. Radioactivity was detected in a phosphorImager using a photostimulable phosphorimaging plate and was analysed by ImageQuant software Version 3.0 (Molecular Dynamics, 240 Santa Ana Court, Sunnyvale).

4.2.2.5. Staining of chromatin using DAPI

Cells were centrifuged at 4,000 x g and fixed by suspension of the pellet in 4% (w/v) paraformaldehyde in phosphate buffered saline (PBS), which contained 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.0 mM $NaHPO_4$. After 1h of fixation at room temperature cells were centrifuged again and washed for 3 x 10 min in PBS at room temperature. Cells were stained by suspension in 0.2 µg/ml DAPI for 5-10 min then placed on slides that had previously been coated with poly-L-lysine (M_r 540,000). Excess liquid was removed, cells were mounted in Citifluor antifade mountant (AFI; City University, London, U.K.) and cover slips were sealed with nail varnish.

4.2.2.6. Staining and observation of preprophase bands of microtubules.

Cells were centrifuged at 5,000 x g, washed twice in 0.4 M mannitol then fixed for 1h in freshly prepared 4% (w/v) paraformaldehyde, 10% (v/v) DMSO 25 mM sodium phosphate adjusted to pH of 9.8 by addition of KOH. Fixed cells were washed twice with 50 mM PIPES, 5 mM EGTA, 2 mM $MgSO_4$ pH 7.4, transferred to a poly-L-lysine treated coverslip and treated for 5 min with 1% cellulysin (from *Trichoderma viride*, Calbiochem), 0.1% pectolyase Y-23 (Seishin Pharmaceutical, Tokyo, Japan), in 0.4 M mannitol, 5 mM EGTA, 50 mM sodium phosphate pH 7.0 freshly supplemented with 1 mg/ml leupeptin and 0.5 mM phenylmethylsulphonylfluoride (MEPI buffer). Cells were then extracted for 15 min in MEPI buffer supplemented with 1% Nonidet P40 and lacking enzymes. After washing with 50 mM sodium phosphate pH 7.0 (3 x 5 min) the cells were incubated overnight with monoclonal anti-β-tubulin (Amersham, Sydney,

Australia) at 1:500 dilution in PBS containing 1% BSA and 0.05% sodium azide. Cells were then washed in PBS (3 x 5 min) and incubated for 2h with FITC-conjugated sheep anti-mouse IgG antibody (Silenus Laboratories, Dandenong, Australia) diluted 1:30, and washed three times in PBS. Nuclei were stained with 0.2 μg/ml DAPI for a few minutes. Observation was as described in chapter 2 for DAPI stained cells, using the standard optiphot FITC filter set for indirect immunofluorescence of β-tubulin. Between 400 and 500 cells were inspected in each sample to determine the percentage with particular cytoskeletal features.

4.3 Results

4.3.1 Okadaic acid and cell proliferation

The old cell line cell suspension culture of tobacco *N. plumbaginifolia* was maintained in standard CSV medium which was diluted at 4 day intervals from a cell density of about 6 x 10^6 per ml to one sixth of that density. Cells, which were diluted in fresh CSV medium and were cultured for 24h, were then treated with 6 μM, 12 μM and 20 μM of okadaic acid respectively for 80 hours. At 6 μM okadaic acid cells remained capable of division and growth, but the rates of cell division and growth were slower than in control cells (Fig. 4.1), indicating at least a partial inhibition of cell division at this concentration. In culture treated with 12 μM of okadaic acid, cell division was completely blocked, as indicated by a constant cell number (Fig. 4.1 and Fig. 4.2.b). In cell cultures treated with 20 μM of okadaic acid, not only was cell division blocked but cell number also declined (Fig. 4.1 and Fig. 4.2.c) indicating lysis caused by a toxic effects of 20 μM of okadaic acid.

4.3.2. Effects of okadaic acid on nuclear events

Okadaic acid at 12 μM resulted in a progressive decline in frequency of cells with G1 content of DNA and a proportionate increase in G2 cells (Fig. 4.4). This shift in frequency indicated that cell cycle progress continued through G1 and S phases until a

specific block to exit from G2 phase was encountered. This correlates with a change in configuration of chromatin and with cytological evidence of a block in early mitosis.

All nuclei of cells treated with 12 μM okadaic acid arrested with a configuration (Fig. 4 3. e) that indicated progress beyond interphase (Fig. 4.3. a) and resembled early prophase because of partially condensed chromatin and of a persisting nucleolus and nuclear membrane (compare Fig. 4.3. b with Fig. 4.3. e). No inhibited cells attained chromosome configurations that corresponded with late prophase or subsequent division stages, which were easily resolved in control cells (Fig. 4.3.c and d). Treatment of cells with 20 μM okadaic acid, which was about twice the minimum concentration required to block division, induced a progression to a more extreme form of chromosome condensation resulting in a granular appearance of the chromatin (Fig. 4.3. f and g). This form of condensation did not resemble normal condensation and it did not lead to metaphase.

The higher concentration (20 μM) of okadaic acid was used to test whether it could induce chromosome condensation in G1 phase. The chromatin of cells treated with 20 μM of okadaic acid for 48h became highly condensed into granular and fragmented configurations (Fig. 4.3. g and h), and finally there was a complete loss of DNA. This was consistent with the decline in the number of cells that could be counted after DNA staining (Figs. 4.1, 4.2.c). Nuclei showing granular and fragmental chromosome condensated showed a single peak of abundance centred on the G2 content of 110 relative units that was identical with the whole population frequency profile (Fig. 4.5). There was no evidence that nuclei had undergone granular chromosome condensation without first replicating DNA and entering G2 phase. These results indicated that 20 μM of okadaic acid had no evident effect on cells passing through G1 to S phases.

4.3.3. Effects of okadaic acid on the cytoskeleton in a short term cell suspension culture of *N. plumbaginifolia*

Presence of PPBs was investigated to test the conclusion, drawn from nuclear structures and DNA content, that cells were arrested in prophase by okadaic acid. PPBs

appear in early G2 phase, mature to a tightly aggregate form by early prophase and therefore provide a marker for cell cycle progress (Gunning and Sammut, 1990). Cells treated with 12 μM okadaic acid at 0, 15, or 19h after release from an aphidicolin block formed preprophase bands that were indistinguishable in appearance from control cells (Fig. 4.6. a and e-g) and formed at an identical time (Fig.4.7). Control cells, which were synchronised with aphidicolin but were not inhibited by okadaic acid, formed PPBs, completed mitosis and formed phragmoplast microtubules between daughter nuclei (Fig. 4.7.a). Culture samples that were treated with okadaic acid at 19h after release from an aphidicolin block included a minority of cells that were insensitive to okadaic acid, probably because they had completed the sensitive steps in mitosis, and these were able to progress to phragmoplast formation. About half of the phragmoplasts formed in the presence of 12 μm inhibitor added at 19h persisted to the end of the sampling period, which was 15h after they had disappeared in control cells and this persistence indicates an inhibitory effect of okadaic acid on completion of phragmoplast function.

The formation of PPBs at the same time and abundance in the population in control and inhibited cells after release from an aphidicolin block (Fig. 4.7) indicated normal rates of progress through the cell cycle to early prophase at 15h, and supported the conclusion drawn from chromatin configuration that prophase was attained. Subsequent behaviour of PPBs in treated cells diverged from normal because disassembly of PPB occurred without the prior occurrence of late prophase and prometaphase events that were blocked by the inhibitor. PPB disassembly in normal cells is accompanied by the formation of an initial mitotic spindle outside the nuclear envelope and its migration into the nucleus as the envelope breaks down allowing alignment of the chromosomes. None of the cells treated with okadaic acid from 0 hour or 15 hour developed an initial extranuclear spindle nor showed any of the later stages of division such as the development of a phragmoplast between daughter nuclei. However, these were detected in control cells (Fig. 4.6. b-d). Disassembly of PPB in inhibited cells was initiated at the same time and proceeded with equal speed as in control cells except that a residual 5% of cells retained PPB up to the end of the sampling period. The reason for this persistence of PPBs in a small minority of cells is not clear. There was no detectable

structural difference between the PPB that persisted (Fig. 4.6.f and g) and the PPB seen at time of their maximum abundance (Fig. 4.6.a).

4.3.4. Effects of okadaic acid on protein phosphorylation

To test whether okadaic acid perturbs the levels of protein phosphorylation control cells and cells inhibited with 12 μM okadaic acid were cultured as in Fig. 4.2 with the addition of $[^{32}P]PO_4$ from 0h and were harvested at 16h when mitotic activity was maximal in the control cells (Fig. 4.2.a). The phosphorylated proteins, which were extracted in the RIPA buffer and resolved on 12 % acrylamide gels and transferred into nitrocellulose, were analysed by PhosphorImager. Net changes in phosphorylation and dephosphorylation of proteins were detected. Okadaic acid at 12 μM resulted in a general elevation of phosphorylation levels among proteins in the range of commonly occurring sizes that was resolved on 12% acrylamide gels. Some size classes of protein were particularly affected since label in proteins of 108 kDa, 49 kDa, 36 kDa, 33 kDa and 31 kDa, after subtraction of adjacent background, was found to be respectively 1.8, 1.25, 1.9, 1.2 and 1.7 fold higher than for the same size classes in control cells. More complex effects of okadaic acid were indicated by a 1.8 fold decline in phosphoprotein at 41 kDa and a 3.3 fold decline in a protein that outside the calibrated size range but of approximately 190 kDa. The time of harvesting samples coincided with a maximum incidence of mitotic cells in the control cells (Fig. 4.2. a), therefore, the greater level of phosphoproteins that were induced by okadaic acid is particularly significant since mitosis coincides with maximum levels of protein phosphorylation.

4.3.5. Effects of okadaic acid on the activity of $p34^{cdc2}$-like protein kinase

The activity of $p34^{cdc2}$-like protein kinase was measured (Fig. 4.9) in control cells and in cells inhibited with 12 μM okadaic acid from 15h to 36h after the release from aphidicolin block. The peak of activity of $p34^{cdc2}$-like protein kinase in inhibited cells occurred 3h earlier at 18h compared with at 21h in control cells. The activity in inhibited cells also reached a slightly higher level of 195 pmole ^{32}P transferred to H1 histone per gram fresh weight cells per min compared with 155 pmole ^{32}P transferred to

H1 histone per gram fresh weight cells per min in control cells. The activity of $p34^{cdc2}$-like protein kinase gradually declined after 18h in the inhibited cells to the end of sampling (36h) showing no evidence of a subsequent cell division cycle. Whereas the activity of $p34^{cdc2}$-like protein kinase in control cells declined after 21h coincident with late mitosis and the appearance of phragmoplasts (Fig. 4.7.a) and then increased from 36h probably due to approach of mitosis in a subsequent cell division cycle. The slightly earlier activation of $p34^{cdc2}$-like kinase correlates with the effect of okadaic acid on $p34^{cdc2}$ in *Xenopus* extracts, in which it also causes an early activation (Haystead et al., 1989; Felix et al., 1990; Lee et al., 1991) that has been attributed to greater phosphorylation and activation of cdc25 phosphatase (reviewed by Murray, 1993).

4.4 Discussion

In animal cells low concentrations of okadaic acid affect the two calcium and magnesium-independent phosphoprotein phosphatases, PP1 and PP2A. Plants have been shown to contain equivalent enzymes at similar levels and with the same sensitivity to okadaic acid (MacKintosh and Cohen, 1989). The observations of effects of okadaic acid on tobacco cell division indicate that normal activities of these calcium and magnesium independent protein phosphatase are necessary for maintenance of normal levels of protein phosphorylation (Fig. 4.8) and for progress through mitosis (Figs. 4.3, 4.4 and 4.7).

When cells were treated with 12 μM of okadaic acid essentially all of the nuclei arrested with partially condensed chromosomes and with persisting nucleoli and nuclear membrane, in a configuration resembling early prophase. It could be concluded that cells progressed through G1 and S phases and were blocked in exit from G2 phase because the frequency of cells in G1 phase declined and a proportional increase of cells in G2 phase occurred (Fig. 4.4). Progress to late G2 phase in the presence of the okadaic acid was also indicated by the development of PPB microtubules synchronously with their appearance in control cells (Fig. 4.7).

It was observed that 20 μM of okadaic acid, which is nearly twice the minimum concentration that is necessary to block all cell division, induced abnormal chromatin configurations. Most nuclei developed a granular form of condensation that was followed by chromosome fragmentation and eventually by complete autolysis of DNA. This progression to chromosome breakdown prevented the accumulation of cells with condensed chromatin and was reflected in the eventual decline to 14% in number of cells with recognisable nuclei. Although it cannot be concluded whether the eventual degradation of chromatin seen with 20 μM of okadaic acid is due to abnormal chromosome structure or an abnormal metabolic state, some disturbance of metabolism is expected since in animal cells a general elevation of protein phosphorylation by okadaic acid, like that seen here, had effects on carbohydrate and lipid metabolism (Haystead et al., 1989). However, metabolism was sufficiently normal, even at 20 μM okadaic acid, to allow all treated cells to progress through G1 and S phases before arresting in G2 phase (Fig. 4.5). One possibility is that a high concentration of the inhibitor induce a higher degree of condensation resembling that of metaphase but restricted to many small areas. The intervening less-condensed region could be more rapidly digested when autolysis began, so resulting in the observed fragmentation of chromosomes.

The unusual condensation of chromatin seen with okadaic acid might result from higher activity of $p34^{cdc2}$-like kinase as indicated in Fig. 4.9. But the evidence in that figure must be interpreted with caution since it could indicate that okadaic acid has had an effect of sharpening synchrony, by holding a greater proportion of cells in the population in prophase with active $p34^{cdc2}$-like kinase, rather than increasing the activation state of $p34^{cdc2}$-like kinase in individual cells.

Although higher concentrations of okadaic acid induced more extreme chromosome condensation it was unable to induce premature chromosome condensation in G1 or S phase. Mitotic animal cells are able to transmit such a signal for condensation on fusion with cells in G1 phase or S phase (Rao and Johnson, 1974) and okadaic acid is able to induce chromosome condensation in hamster cells, but not in mouse or human cells (Steinmann et al., 1991). In tobacco, okadaic acid treated cells

progressed from G1 phase to G2 phase prior to the maximum abundance of nuclei with condensed chromatin (Fig.4.2).

The present evidence that processes sensitive to okadaic acid make an essential contribution to intranuclear events in plant mitosis is consistent with genetic evidence in fungi that the *bim* G mutation of *Aspergillus* (Doonan and Morris, 1989; Ohkura et al., 1989) and *dis2/bws* mutation of fission yeast (Booher and Beach, 1989), which both block mitosis, are in genes with deduced products that are similar to mammalian protein phosphatase type 1. These mutations prevent normal anaphase and therefore cause a slightly later block than that in tobacco. This difference need not indicate that phosphatases have different functions in fungal and plant mitosis since the earlier arrest in plant cells would have masked any later block point equivalent to that seen in the mutant fungi and therefore the possibility that later stages of plant mitosis are also sensitive to okadaic acid cannot be eliminated. The difference between the late mitotic arrest seen in mutant yeasts and the prophase arrest seen in these inhibited tobacco cells may derive from the type of phosphatase enzyme affected. The mutations are predicted to affect protein phosphatase type 1 but okadaic acid is more likely to affect type 2A, which is ten times more sensitive to it (Gschwendt et al., 1989), although effects of the okadaic acid on type 1 phosphatase cannot be ruled out (Cohen, 1989; Cohen et al., 1989).

The development of PPBs with equal frequency and timing in cells inhibited with okadaic acid and in control cells (Fig.4.7) provided further evidence that G2 phase was attained in inhibited cells. PPBs appear in early G2 phase and develop to a mature tightly aggregated state by early prophase (Gunnning and Sammut, 1990). Their configuration in inhibited cells was indistinguishable from those in control cells at early prophase (Fig. 6)

Behaviour of PPBs provided unexpected evidence that the occurrence of some mitotic events can be uncoupled from the occurrence of events that normally precede them. In 95% of inhibited cells PPBs progressed to disassembly at the same time as in control cells (Fig.4.7) although nuclear events had not progressed beyond early prophase. In normal plant cell division and in the control cells studied here PPB disassembly occurs at the beginning of metaphase, as a nuclear spindle forms outside the

nucleus and the nuclear membrane breaks down allowing access of the spindle to the nucleus and the establishment of metaphase. The dissociation of PPB breakdown from the occurrence of these events has not been found before.

It is tempting to speculate on the identity of the signal for PPB breakdown. Nuclear displacement experiments have indicated that a positive signal from an adjacent metaphase nucleus is necessary to trigger PPB breakdown (Murata and Wada, 1992). It is possible that an increase in phosphorylation state of a PPB protein(s) is part of the normal signal for disassembly and that the increase is resistant to concentrations of okadaic acid that disrupt the interactions of protein kinases and protein phosphatases within the nucleus that drive chromosome condensation, spindle assembly and nuclear envelope break down. This hypothesis is supported by the observation that activity of $p34^{cdc2}$-like protein kinase activity, which binds to $p13^{suc1}$-beads and is eluted by free $p13^{suc1}$, is fully resistant to okadaic acid inhibition (Fig. 4.9) and could therefore provide the PPB breakdown signal. It is also supported by the observation of putative protein kinase in PPBs by reaction with monoclonal antibody specific for the PSTAIR protein (Mineyuki et al., 1991). Proteins containing the PSTAIR amino acid sequence are related to the protein kinase $p34^{cdc2}$, which is capable of altering the cytoplasmic cytoskeleton of animal cells (Lamb et al., 1990). The available evidence is therefore consistent with involvement of this class of protein kinase in PPB disassembly since this kinase activity is activated in control and inhibited cells at the time of PPB breakdown. The hypothesis that preprophase band breakdown could be triggered by $p34^{cdc2}$ activation is further supported by the recent observation that staurosporine, which is a protein kinase inhibitor that inhibits animal $p34^{cdc2}$ can prevent PPB breakdown (Katsuta and Shibaoka, 1992).

An hypothesis to account for the effect of okadaic acid on tobacco cell division is that it disturbs an ordered sequence of phosphorylation and dephosphorylation of proteins that is essential for mitosis. The accumulation of higher general levels of phosphoproteins and of some phosphoproteins in particular is consistent with the possibility that proteins are normally phosphorylated transiently during division but accumulate in phosphorylated forms in the presence of the phosphoprotein phosphatase

inhibitor. The few phosphoproteins that were at decreased levels in the inhibited cells may be phosphorylated during the later stages of mitosis that were not reached in the inhibited culture, or may be substrates of protein kinases that were inactivated by phosphorylation (Gould and Nurse, 1989) that was accentuated by okadaic acid. As discussed above, phosphorylated proteins are more abundant at mitosis in animals cells (Maller and Smith, 1985; Karsenti et al., 1987) and in the unicellular plant *Chlamydomonas* (Harper et al., 1990). The present observation of specific arrest at mitosis by okadaic acid indicates that protein phosphorylation is not only more abundant but is also more critical to cell cycle progress at mitosis than in earlier cell cycle phases.

Hasezawa and Nagata (1992) have suggested that in tobacco BY-2 cells okadaic acid-sensitive cell cycle transitions occur at 3 points; at the border of M/G1 phase, before the formation of the preprophase band, and at anaphase. The data of Hasezawa and Nagata (1992) indicate that BY-2 cells are more sensitive to okadaic acid than the *N. plumbaginifolia* cells since they observed strong inhibition at 1 μM, although they do not report investigation of any lower concentrations. When transferring cells from synchronous culture to 1 μM of okadaic acid they observed that cells progress little, or not at all, from their cell cycle location at time of first exposure to okadaic acid. Thus cells in G1 phase failed to enter S phase, cells in G2 phase failed to form PPBs or enter mitosis and cells in anaphase failed to form phragmoplasts. It may be that the concentration of 1 μM of okadaic acid used by Hasezawa and Nagata, although lower than that used in the current study, was excessive because BY-2 cells are more sensitive. The concentration used by Hasezawa and Nagata (1992) failed to detect any differences in sensitivity to the inhibitor between different cell cycle events. From their evidence it is impossible to eliminate the possibility that the inhibitor was causing a general disruption of housekeeping metabolism in all cycle phases. Hasezawa and Nagata (1992) chose to interpret their data in terms of an inhibition of the next scheduled cytoskeletal change in each cycle phase, but there is no direct evidence to support that view given the universal inhibition of cycle progress in all phases of the cell cycle that they observed. It would require prohibitively large amounts of okadaic acid to attempt to repeat their effects using *N. plumbaginifolia* but it would be interesting to test lower concentrations of

okadaic acid than used by Hasezawa and Nagata with *N. tabacum*. My results predict that there would then be a preferential inhibition of mitosis, indicative of a greater importance of protein phosphorylation at that time.

Fig. 4.1.

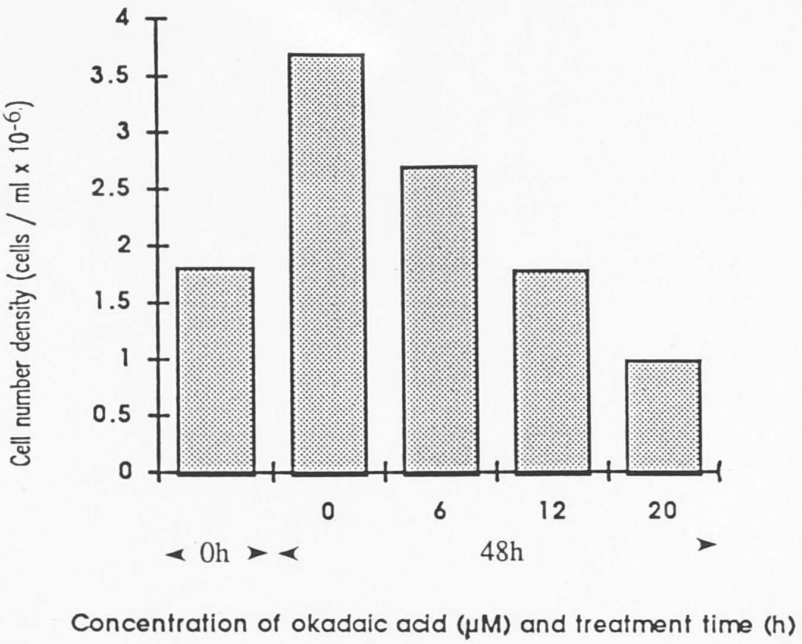


Fig. 4.1 Effect of okadaic acid on increase in cell number in suspension cultures. Initial cell number at 0h can be compared with number observed after 48h treatment with 0.4% DMSO and, (0) zero or (6), (12), (20) respectively 6, 12, 20 μM okadaic acid.

Fig. 4.2 Cell number density and chromatin configuration in cultures treated with: (a) DMSO without okadaic acid; (b) DMSO and 12 μM okadaic acid; (c) DMSO and 20 μM okadaic acid. Symbols indicate: (○) cell number / ml culture; (△) percentage of cells in anaphase; (□) percentage of cells with early prophase-like nuclear configuration; (●) percentage of cells with granular chromatin; (▲) percentage of cells with fragmented chromatin.

Fig. 4.2.

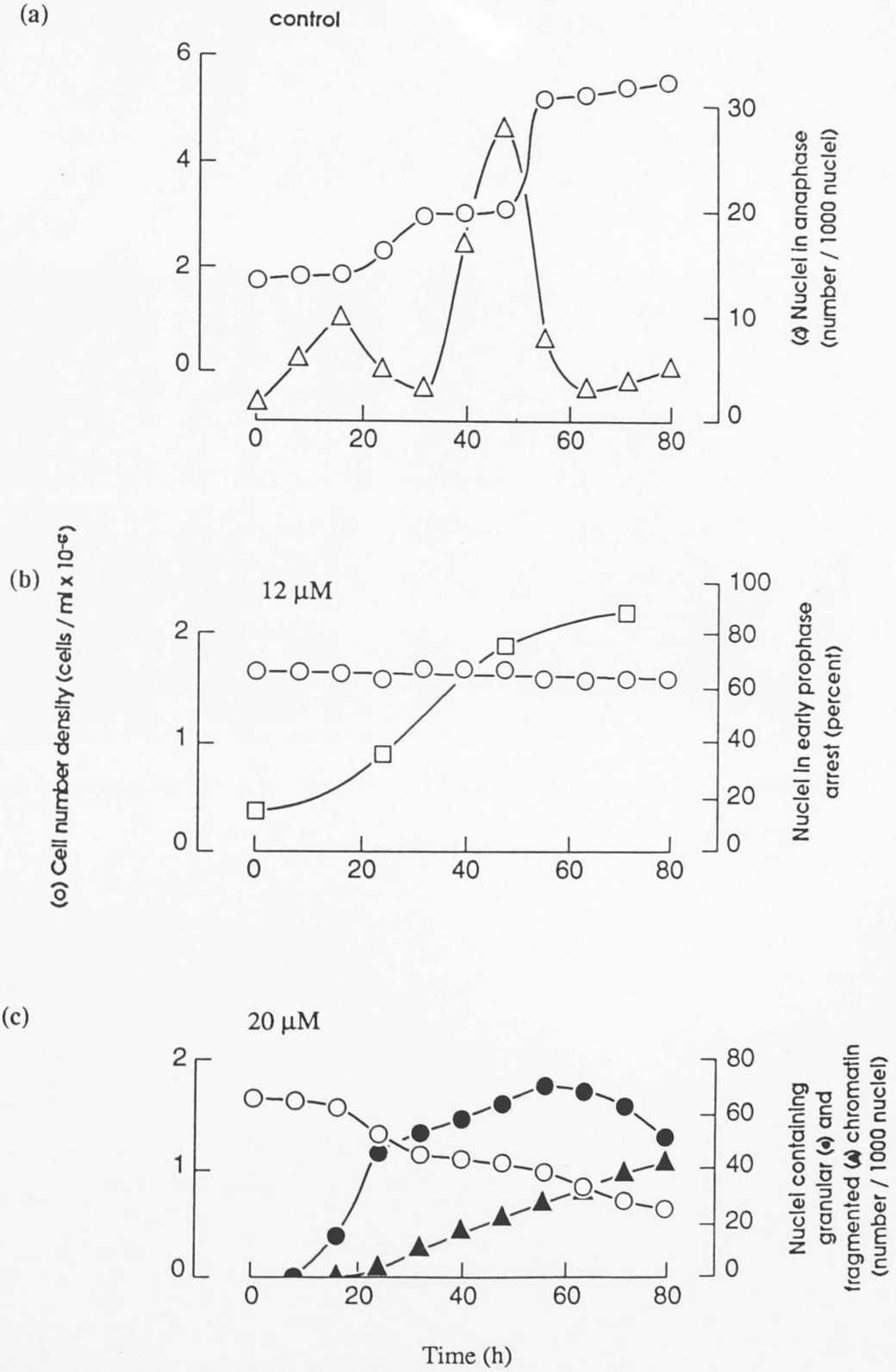

(a)
control
6
4
2
0
30
20
10
0
(Δ) Nuclei in anaphase (number / 1000 nuclei)
0
20
40
60
80
(b)
12 μM
(o) Cell number density (cells / ml x 10⁻⁶)
2
1
0
100
80
60
40
20
0
Nuclei in early prophase arrest (percent)
0
20
40
60
80
(c)
20 μM
2
1
0
80
60
40
20
0
Nuclei containing granular (•) and fragmented (▲) chromatin (number / 1000 nuclei)
0
20
40
60
80
Time (h)

Fig. 4.3 Chromatin configurations revealed by DNA staining in (a-d) uninhibited control cells and (e-f) cells treated with okadaic acid. Uninhibited cells progressed through: (a) interphase; (b) early prophase; (c) late prophase; (d) anaphase. Cells treated with 12 μM okadaic acid accumulated in a configuration very similar to early prophase as seen in (e) which shows a treated cell at 48 h. The nucleolus is arrowed in (a), (b) and (e). Cells inhibited with 20 μM okadaic acid showed a more extreme granular condensation of chromatin, illustrated in cells seen after 48 h treatment (f, g) and progressed to chromatin fragmentation (h).

Fig. 4.3.

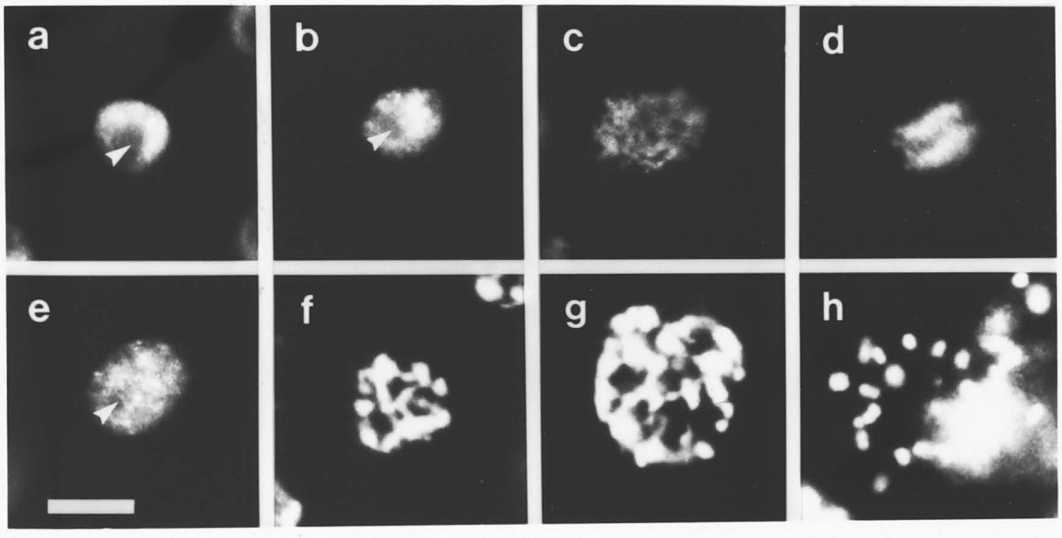


Fig. 4.4 Change in frequency of nuclear DNA contents in a culture treated with 12 μM okadaic acid. The profile of the initial population is shown in (a) and the change in frequency, relative to the initial population, that was observed at 24 h, 48 h and 72 h is shown in (b), (c) and (d) respectively. Decrease in frequency is indicated by open bars and increase in frequency by stippled bars.

Fig. 4.4.

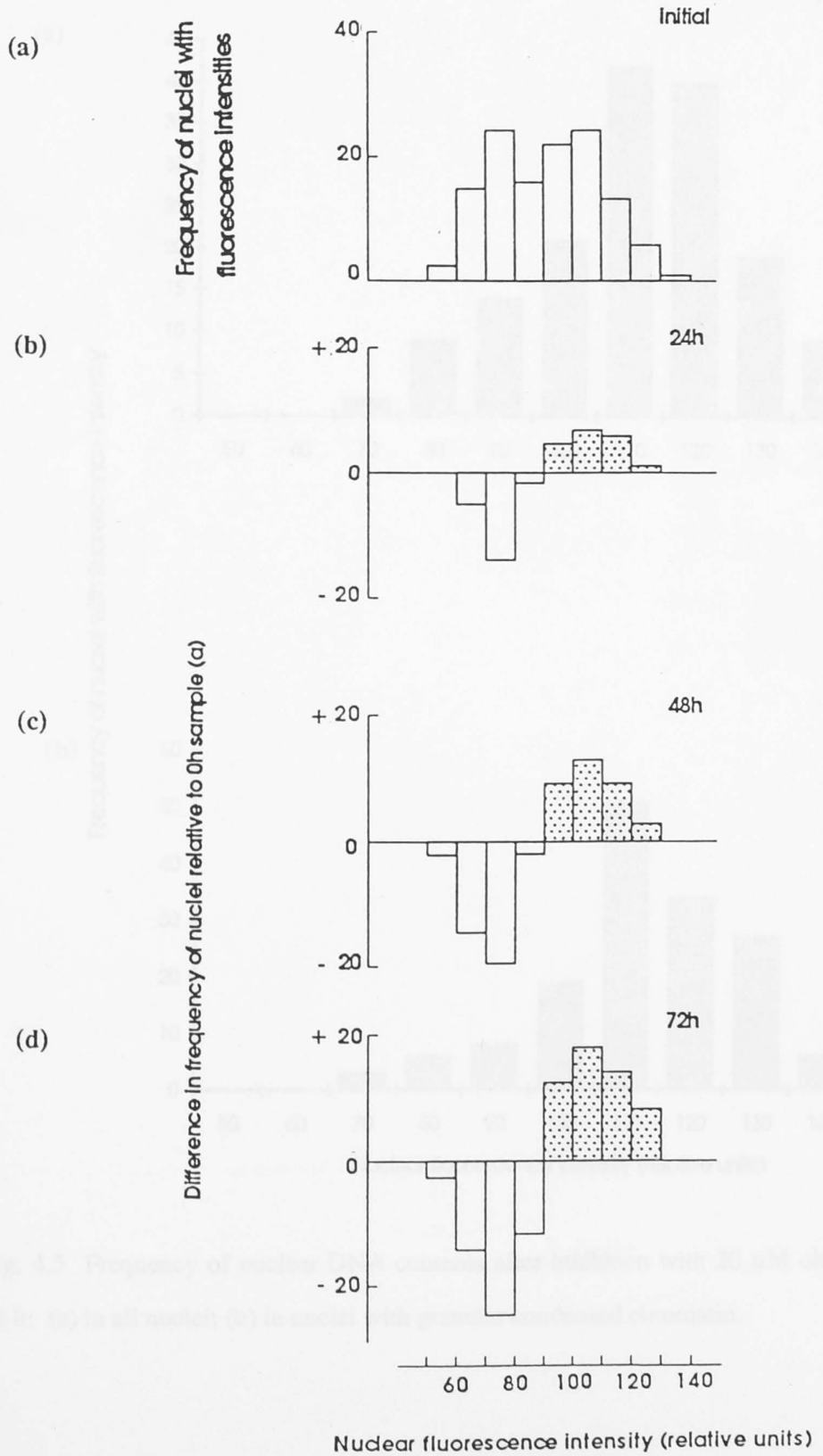

(a)
Initial
40
20
0
Frequency of nuclei with fluorescence intensities
(b)
24h
+ 20
0
- 20
(c)
48h
+ 20
0
- 20
(d)
72h
+ 20
0
- 20
Difference in frequency of nuclei relative to 0h sample (a)
60
80
100
120
140
Nuclear fluorescence intensity (relative units)

Fig. 4.5.

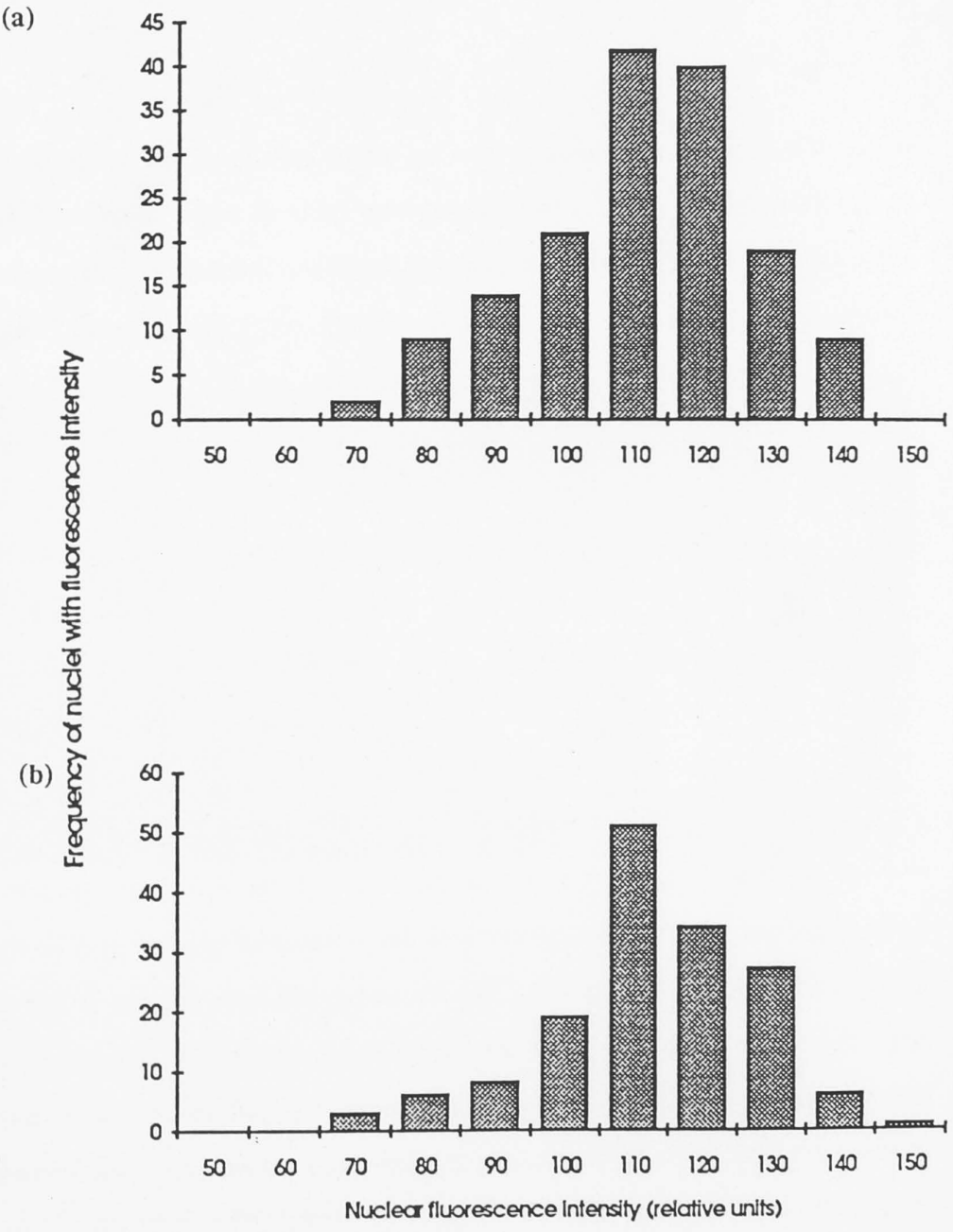


Fig. 4.5 Frequency of nuclear DNA contents after inhibition with 20 μM okadaic acid for 48 h: (a) in all nuclei; (b) in nuclei with granular condensed chromatin.

Fig. 4. 6 Division in synchronously dividing control cells (a-d) and in cells treated with 12 μM okadaic acid (e-h). DNA stained with DAPI is shown in (d) and (h), other images show indirect immunofluorescence of β-tubulin. Frequency of preprophase band (PPB) microtubules in these cultures is shown in the accompanying Figure 7. (a) shows a mature PPB that is characteristic of early prophase that was seen in the uninhibited culture at 20 h and (b) shows the prometaphase configuration that normally immediately precedes PPB disassembly, in which an initial mitotic spindle has formed outside the nucleus while the PPB is still present. This prometaphase cell was observed at 21h in the control culture and illustrates the earliest stage of normal mitosis that was not reached by cells in the inhibited cultures. Further progress in the control culture lead to formation of a phragmoplast separating daughter nuclei (c, d, double label of same cell), which also was not seen in inhibited cells. (e) shows a mature PPB that formed in a cell treated with okadaic acid from 0h sampled at 21h. It is typical of PPBs formed in the presence of the inhibitor and is indistinguishable from normal PPB. (f) and (g) show examples of the small proportion of PPBs that persisted in the presence of okadaic acid whether the inhibitor was added at 0, 15 or 19h. The illustrated cells were sampled at 24h and 39h respectively from a culture to which okadaic acid was added at 19h. Most inhibited cells disassembled their PPBs but they did not progress beyond the extent of chromatin condensation typical of early prophase (h) and none showed complete chromosome condensation.

Fig. 4.6.

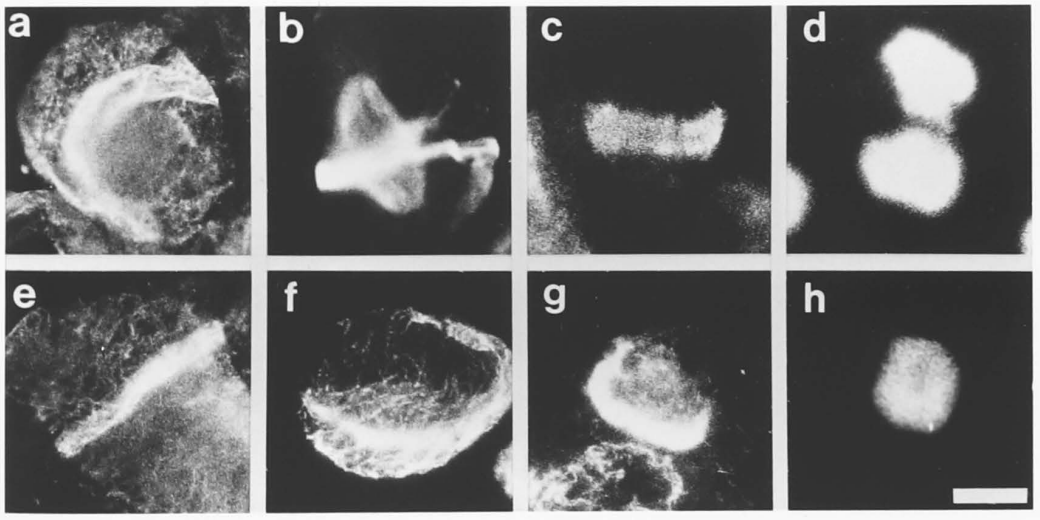


Fig. 4.7 Frequency of (○) preprophase band and (Δ) phragmoplasts in synchronised cultures treated with (a) DMSO only from 0h; (b) DMSO and 12 μM okadaic acid from 0h; (c) DMSO from 0h and 12 μM okadaic acid from 15h; (d) DMSO from 0h and 12 μM okadaic acid from 19h. DMSO was at 0.4% in each case.

Fig. 4.7.

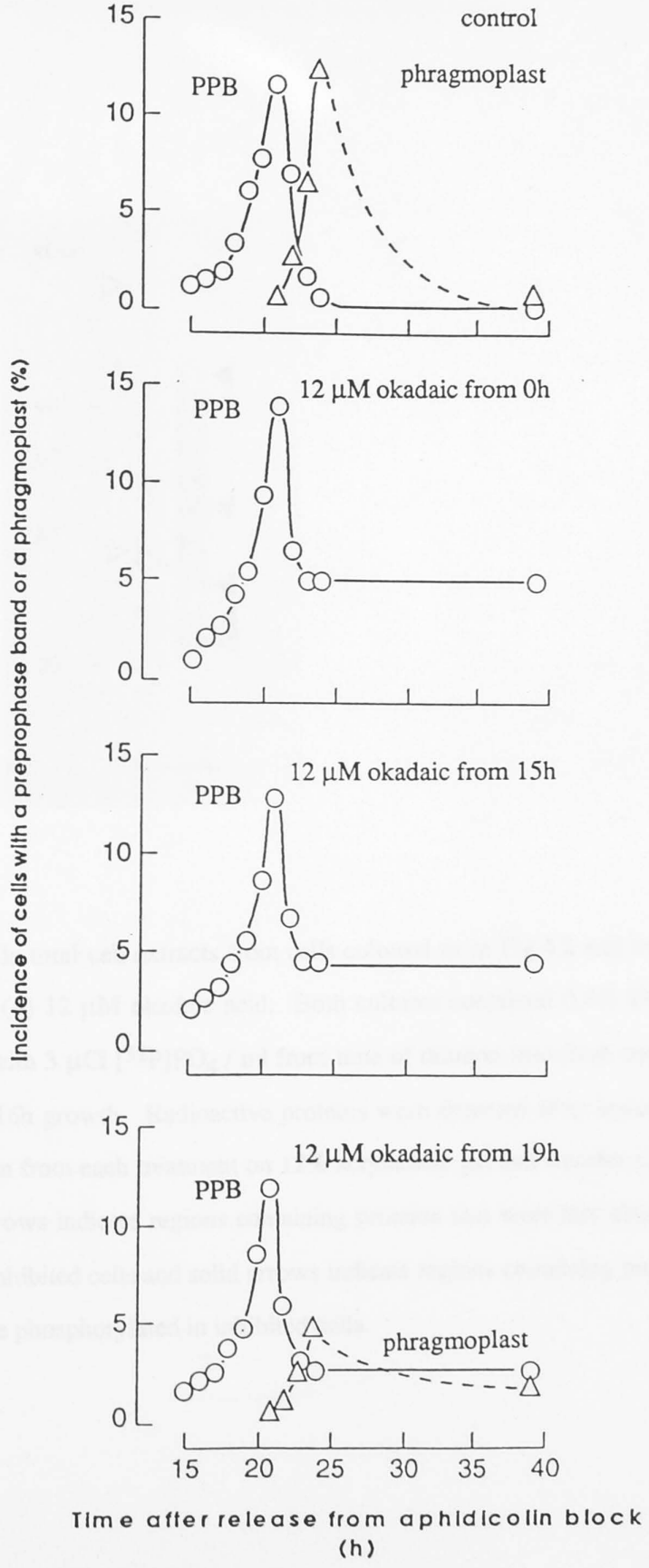

(a)
control
PPB
phragmoplast
(b)
12 μM okadaic from 0h
PPB
(c)
12 μM okadaic from 15h
PPB
(d)
12 μM okadaic from 19h
PPB
phragmoplast
15
10
5
0
15
20
25
30
35
40
Incidence of cells with a preprophase band or a phragmoplast (%)
Time after release from aphidicolin block (h)

Fig. 4.8.

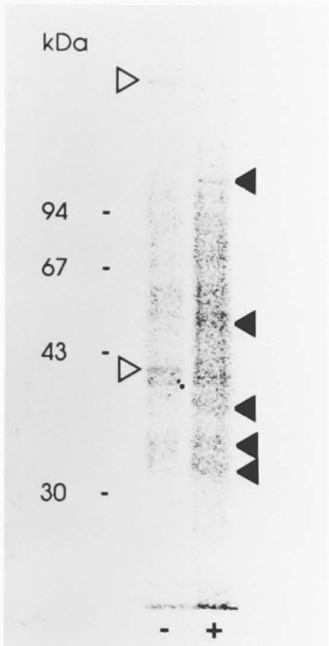
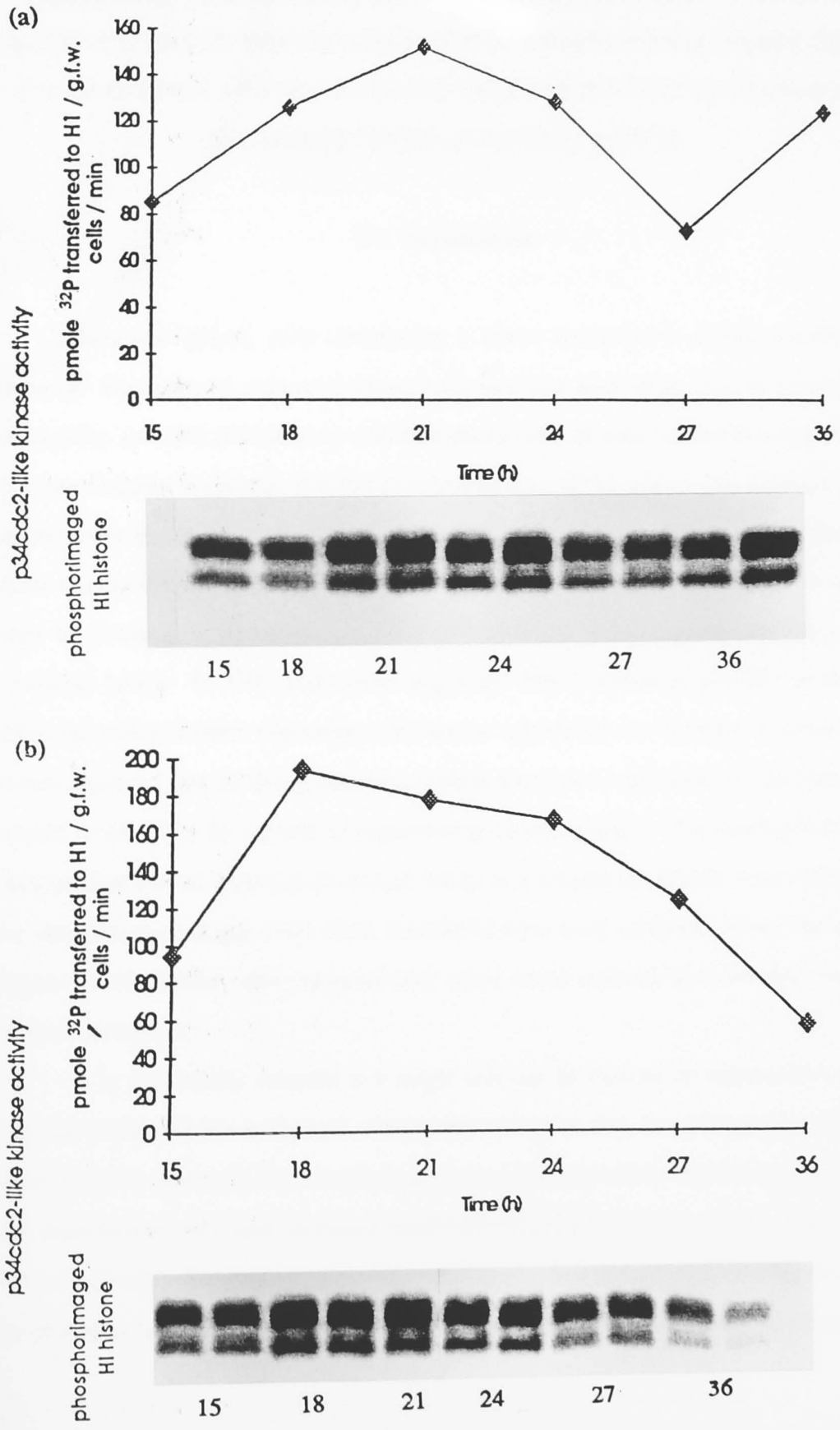


Fig. 4.8 Phosphoproteins in total cell extracts from cells cultured as in Fig.4.2 and treated with; (-) no okadaic acid; (+) 12 μM okadaic acid. Both cultures contained 0.4% DMSO and cells were incubated with 3 μCi $[^{32}P]PO_4$ / ml from time of dilution into fresh medium and were harvested after 16h growth. Radioactive proteins were detected after separation of 50 μg samples of protein from each treatment on 12% acrylamide gel and transfer to 0.1 μ nitrocellulose. Open arrows indicate regions containing proteins that were less abundant or less phosphorylated in inhibited cells and solid arrows indicate regions containing proteins that were abundant or more phosphorylated in inhibited cells.

Fig. 4.9 Activity of $p34^{cdc2}$-like protein kinase in the cells treated with DMSO-only (0.4 % of v / v) from 15 h to 36 h (a); DMSO (0.4 % of v / v) and 12 μM okadaic acid from 15 h to 36 h (b). The $p34^{cdc2}$-like protein kinase was purified from 0.1g of cell powder in NDE buffer by 20 μl of $p13^{suc1}$ beads and eluting with 50 μl of 0.5 mg / ml of $p13^{suc1}$ solution. The activity of $p34^{cdc2}$-like protein kinase was measured using histone H1 as substrate at 30°C for 5 min. The amount of ^{32}P transferred to H1 histone was measured by placing 20 μl of reaction mixture on p81 phosphocellulose paper washing with 75 mM phosphoric acid and counting in a scintillation counter; different ordinate scales are used in presenting counts from control and inhibited cells. PhosphorImaged histone H1 was obtained by separating 30 μl of reaction mixture on 12% acrylamide gel and exposure in a phosphorImager.

Fig.4.9.


(a)
p34cdc2-like kinase activity
pmole 32P transferred to H1 / g.f.w. cells / min
0
20
40
60
80
100
120
140
160
15
18
21
24
27
36
Time (h)
phosphorimaged H1 histone
15
18
21
24
27
36
(b)
p34cdc2-like kinase activity
pmole 32P transferred to H1 / g.f.w. cells / min
0
20
40
60
80
100
120
140
160
180
200
15
18
21
24
27
36
Time (h)
phosphorimaged H1 histone
15
18
21
24
27
36

CHAPTER 5

CHANGES IN THE LEVEL OF p34^{cdc2}-LIKE PROTEIN AND ITS ENZYME ACTIVITY, AND IN THE OCCURRENCE OF PREPROPHASE BANDS, SEEN IN LONG-TERM AND SHORT-TERM CELL SUSPENSION CULTURES OF *NICOTIANA PLUMBAGINIFOLIA* (NPT 5)

5.1 Introduction

In higher plants, cells constituting a tissue operate in a highly coordinated manner. However, an organised tissue, such as a root or a stem, may be completely changed to a rapidly proliferating undifferentiated mass of cells (callus) if cultured on a nutrient medium containing specific phytohormones such as auxins (for example IAA, or the more stable analogues 2,4-D, or NAA), and cytokinins (for example zeatin or more usually the analogues kinetin or BAP). From such callus, cell suspension cultures may be obtained if transferred into a liquid medium containing suitable auxin and cytokinin levels. In 1902 Haberlandt suggested that it should be possible to culture artificial embryos from vegetative cells and he introduced the concept of totipotency which proposed that all living cells that contain a normal complement of chromosomes should in principle be capable of regenerating an entire plant. The starting point for regeneration may be a portion of excised tissue, or a suspension culture, since callus can be obtained from single cells when transferred onto solid medium. From the callus organogenesis and/or embryogenesis may occur when induced by a suitable ratio of auxin to cytokinin.

It is particularly valuable if a single cell can be induced to regenerate a plant because if the cell has integrated a beneficial new gene into its genome the resulting plant will homogenously contain the desired new character and it will be represented in the reproductive cells and therefore uniformly propagated to succeeding generations. Such single cells that may be suitable for the introduction of new genes can be obtained by protoplasting either suspension culture cells or tissues. The protoplast opens the

possibility of genetic transformation by electroporation or by use polyethylene glycol. A particularly convenient source of cells for protoplasting is the suspension culture, from which cells can be obtained in sterile form at any time and in a dispersed state very suitable for cell wall removal. The recent technique of biolistic bombardment of unmodified plant tissue is proving valuable but it still carries the disadvantage that only some cells in a bombarded tissue will have been penetrated and only a few of these will have integrated any introduced gene; therefore causing considerable problems in recovering ovules or pollen containing the introduced gene. If a selectable marker, such as resistance to an antibiotic, is introduced together with the gene for a desired new character then transformed individual cells derived from protoplasts can be effectively selected. Selection is less effective when applied to bombarded tissue, since the selective agent may not penetrate fully at effective concentrations and if many surrounding cells are killed the transformed cell may not survive.

Unfortunately the greater experimental convenience of deriving cells from suspension culture and the advantage of more easily avoiding chimeras is limited by changes in regenerability that occur in suspension cultures.

Early in the 1950's, it was found that plant cells in culture may lose the capacity for morphogenesis. For example, tobacco tissue after prolonged sub-culture loses the capacity to form organs (Kehr and Smith, 1954; Murashige and Nakano, 1965), several cultivars of carrot showed that the loss of embryo-formation occurred with high frequency after culture for 30-40 weeks (Reinert et al., 1968, 1970) similarly, abnormal morphological development occurred in regeneration from callus tissue of tobacco after it had been propogated for a long time (Melchers, 1966). This loss of morphogenetic capacity involves to the loss of regeneration of either buds or roots or both (Murashige and Nakano, 1965; Syono, 1965; Gautheret, 1966). The loss of morphogenetic ability by callus correlates with some physiological characteristics, for example tobacco tissue which lost the capacity to form organs after prolonged culture had become habituated to growing without added auxin (Gautheret, 1948). So the loss of morphogenesis in tissue and callus culture is a common phenomenon in cell suspension and tissue cultures from higher plants.

Since the 1960's, people have attempted to explore the mechanisms underlying the loss of morphogenetic capacity from cultured cells. Most work concentrated on analysis of changes in chromosomes. For example callus from tobacco *Nicotiana tabacum* L. Wisconsin 38 pith cells originally contained nearly equal proportions of diploid and tetraploid cells when taken from 3.5-10.5 cm below the stem apex and predominantly tetraploid cells with only about 5% of aneuploid cells when taken from 15.5-22.5 cm below the stem apex. But after 6 years culture *in vitro*, capacities for organogenesis gradually declined and correlated with an increasing proportion and eventually a majority of aneuploid cells (Murashige and Nakano, 1967). Similar correlations of declining capacity for morphogenesis with increased incidence of unstable aneuploid cells was found in long-term callus/tissue cultures of pea (Torrey, 1967) and tobacco (Sacristan and Melchers, 1969; Zagorska et al., 1974). Aneuploid changes in chromosome number represent genetic changes that are immediately visible but it is likely that mutational changes, which are not visible at the whole chromosome level, also accumulate in culture and are not selected against if they affect genes required for development but not for simple disorganised proliferation.

The evidence therefore suggests that an accumulation of genetic changes that are un-penalised during artificial culture in the laboratory remove the capacity for regulation and spatial organisation of cell proliferation. There is relatively little understanding of the subcellar and molecular changes that are associated with, and possibly the cause of, loss of regenerability. An absent or an irregular preprophase band of cortical microtubules was observed to correlate with loss of organised morphogenesis in tobacco cell lines (Gorst et al., 1986). In the present investigation the presence of preprophase bands in the short and long term cultured cells was therefore investigated and, because of the correlation of $p34^{cdc2}$ regulation with cell proliferation control noted elsewhere in this thesis, parallel investigations were made of $p34^{cdc2}$-like protein level and enzyme activity. The long-term cell suspension culture of *N. plumbaginifolia* (NPT 5), which has been cultured for more than 5 years, can form only disorganised callus on solid medium having no ability to regenerate organs, whereas the

short-term cell suspension culture of tobacco (NPT 5) that had only been sub-cultured for 5 months still had the ability to regenerate organs.

5.2. Materials and methods

5.2.1 Materials

A cell suspension culture of *N. plumbaginifolia* (NPT 5), which had been cultured for more than 5 years and kindly provided by Dr Philip J Larkin, CSIRO, was used as long-term cell suspension culture, and for comparison a short term suspension culture was established by transfer of leaf callus to liquid medium (described in Chapter 4) and was analysed at five months after transfer to liquid medium, when the culture had just begun to be evenly dispersed but was still predominantly in groups of around 30-50 cells and could still regenerate shoot and root tissues.

5.2.2. Methods

5.2.2.1. The methods for preparation of a short-term cell suspension culture and for the observation of preprophase bands of microtubules were the same as in chapter 4. The methods of measurements of level and kinase activity of $p34^{cdc2}$-like protein were the same as in chapter 2.

5.2.2.2. Test of regenerability of tobacco cell suspension culture

1. Testing media

The eight different combinations of auxin and cytokinin were used to test for regeneration of tobacco suspension cultures. These were MS medium containing 1 mg/l IAA and 0.2 mg/l BAP (A), 0.5 mg/l IAA and 0.2 mg/l BAP (B), 1 mg/l IAA and 0.1 mg/l BAP (C), 0.5 mg/l IAA and 1 mg/l BAP (D), 1 mg/l IAA (E), 0.2 mg/l BAP (F), hormone-free MS (G) and CSV medium containing 2 mg/l 2,4-D and 0.05 mg/l kinetin.

2. Test of regenerability

2 ml aliquot of short-term or of long-term cell suspension culture were spread on the surface of plates containing A, B, C, D, E, F, G and CSV solid medium

respectively and then incubated at 25°C-26°C with 8 hour light and 16h dark for 2-4 weeks and inspected for the appearance of shoots and/or roots.

5.3 Results

5.3.1. Establishment of a regenerable cell suspension culture of tobacco *N. plumbaginifolia*

Callus of tobacco *N. plumbaginifolia* was induced when tissue was taken, either from the first 7 leaves below the stem apex or by excision of stem pith, and cultured on solid MS medium containing either 1 mg/l NAA and 0.4 mg/l BAP or 1 mg/l NAA and 1 mg/l BAP. Fig. 5.1 shows callus derived from leaves that was induced by (A) 1 mg/l of NAA and 1 mg/l of BAP or (B) 1 mg/l of NAA and 0.4 mg/l of BAP. The short-term cell suspension culture, shown in Fig. 5.2, was obtained when the callus from leaves was transferred into either liquid MS or CSV medium containing 2 mg/l of 2,4-D and 0.05 mg/l of kinetin and cultured for about 5 months. This cell suspension culture was used to compare with long-term cell suspension culture in their capacity to differentiate into organs.

5.3.2. Comparison of the ability to regenerate shoot buds or roots between long-term and short-term cell suspension cultures

The long-term cell suspension culture could only divide and grow to form callus without any shoot or root initiation when the cell suspension culture was cultivated on inducing media A, B, C, D, E, F, G (described in 5.2.2.2.) and complete CSV medium for 4 weeks respectively. Fig. 5.3 shows the callus from long-term cell suspension culture which was grown on (A) CSV medium containing 2 mg/l of 2,4-D and of 0.05 mg/l of kinetin and (B) MS medium containing 0.5 mg/l IAA and 1 mg/l BAP.

Not only callus but also differentiation of buds could be induced when cells from the short-term cell suspension culture were transferred to inducing media. The best bud organogenesis was observed in callus that was cultivated on MS medium containing 0.5 mg/l of IAA and 1 mg/l of BAP (Fig. 5.4-A). Fig. 5.4-B shows bud

differentiation induced by 1 mg/l of IAA and 0.1 mg/l of BAP and Fig. 5.4-C shows bud initiation induced by 2 mg/l of 2,4-D and 0.05 mg/l of kinetin.

5.3.3. Occurrence of preprophase bands of microtubules in long-term and short-term cell suspension cultures of tobacco *N. plumbaginifolia*

To maximise the effectiveness of observations aimed at detecting the presence of preprophase bands synchronously dividing cultures were employed since this cytoskeletal element is present only during G2 phase and therefore is present in only minority of asynchronous cells. Cells arrested with aphidicolin are blocked in S phase and on release soon enter G2 phase, however no preprophase bands of microtubules could be detected in the G2 phase of a long-term suspension culture that had been synchronised by aphidicolin when it was sampled at every hour after release from aphidicolin block. Cells were fixed in paraformadehyde and double-stained with the antibody against β-tubulin and with DAPI. Another microtubule-containing cytoskeletal element, the phragmoplast, could be observed with a peak of occurrence at 22h that correlated with synchronous division. However, in short-term cell suspension culture, not only phragmoplasts but also preprophase bands could be observed. The peak of preprophase band occurrence was at 20h after release from aphidicolin block and the peak of phragmoplast occurrence was 2h later at 22h reflecting progress from mitosis to cytokinesis in the synchronised cells (Fig. 5.5.b and Fig. 5.6). Fig. 5.6.a-g shows the preprophase bands of microtubules observed in the short-term suspension culture and Fig. 5.6.h shows the appearance of phragmoplasts, which could be observed in both the long-term and the short-term suspension cultures and demonstrated uniformity of microtubule immunostaining. It was noted that both loss of preprophase bands and loss of the ability to regenerate organs had occurred in the long-term cell suspension culture while the short-term cell suspension culture, which still had the ability to regenerate organs, had retained preprophase bands of microtubules.

5.3.4. Changes of protein level and catalytic activity of p34^{cdc2}-like protein kinase in long-term and short-term cell suspension cultures of tobacco *N. plumbaginifolia*

Possible changes in level and activity of p34^{cdc2}-like protein kinase through the cell cycle were investigated in long-term and short-term cell suspension cultures. Cell division was synchronised with aphidicolin and the resulting peaks of S phase activity, which were detected by BrdU incorporation, are shown in Figs. 5.8.a and 5.9.a. For assay of p34^{cdc2}-like protein levels 0.1 g of frozen cell powder of each sample were extracted in RIPA buffer, separated in 12% SDS-acrylamide gels, transferred to nitrocellulose for Western blotting and probed with affinity purified antibody against PSTAIR peptide then with a second antibody ^{125}I-anti-rabbit-IgG. A striking fourfold difference in level of p34^{cdc2}-like protein per 50 µg of total protein was detected, with long-term cultured cells containing around 16 x 10^6 units throughout the cell cycle while short-term cultured cells contained around 4 x 10^6 units (Fig. 5.7).

The difference in level of p34^{cdc2}-like protein between the long-term and short-term suspension cultures is also partially reflected in the level of kinase activity. Although the peak time of activity of p34^{cdc2}-like protein kinase in both the long-term and short-term suspension cultures was at 19h after release from aphidicolin block (Figs. 5.8.b, 5.9.b) the peak levels of p34^{cdc2}-like protein kinase activity in the long-term suspension culture was about 3 times higher than in the short-term suspension culture (Figs. 5.8.b, 5.9.b), as indicated by about 58 pmoles ^{32}P transferred to H1 histone/ g.f.w. cells/ min at 19h in long-term suspension culture and about 18 pmoles ^{32}P transferred to H1 histone / g.f.w. cells / min at 19h in short-term suspension culture (Figs. 5.8.b and 5.9.b).

Therefore not only the level but also the activity of p34^{cdc2}-like protein kinase was considerably higher in the long-term cell suspension culture that had lost the ability to regenerate organs than in short-term cell suspension culture, which still had the ability to regenerate organs.

5.4. Discussion

5.4.1. Occurrence of preprophase bands of microtubules and capacity for organogenesis in cell suspension cultures

Preprophase bands occur widely in higher plants, for example, in root and shoot meristems, and in cambium, leaf tissue and uniseriate trichomes as well as in cultured 2- and 3-dimensional aggregates of cells (Pickett-Heaps, 1966; Gunning et al., 1978; Busby and Gunning, 1980). It is normally accepted that the preprophase band of microtubules is a key developmental determinant in plant cells since it marks the place where the future cross wall will join with the existing mother cell wall and it therefore plays a part in determining the placing of new cells (Gunning et al., 1978). Preprophase bands are also found during regeneration of vascular tissue around a wound in pea roots (Hardham and McCully, 1982). However, there is no preprophase band in some higher plant cells. They are absent, for example in endosperm tissue and this correlates with its lack of specific internal organisation (Wick and Duniec, 1984).

In the long-term culture of tobacco cells studied here absence of preprophase bands correlated with the loss of the ability to regenerate organs. It is possible that the occurrence of preprophase bands indicates "developmental order" (Fowke and Gamborg, 1980; Vander Valk et al., 1980; Lloyd, 1982; Wick and Duniec, 1984) which is necessary for organogenesis. The observation by Gorst et al (1986) that the low PPB index and irregular PPBs in tobacco suspension culture lines correlated with their inability to undergo organised morphogenesis, accords with the results in this section. However, they also observed that the occurrence of preprophase bands at a relatively high PPB index (ratio of PPB to phragmoplasts containing cells) may be observed in both embryogenic and nonembryogenetic carrot cell suspension cultures, and pointed out that a high PPB index is necessary for embryogenesis but is not obligatorily coupled to it.

5.4.2. Changes of p34^{cdc2}-like protein kinase level and the capacity for organogenesis in cell suspension cultures of tobacco

The preprophase band is a readily-detectable structural element that has been proposed as a useful indicator of a capacity to regenerate Gorst et al., 1986). Since the PPB determines the plane of cell division it has a likely role in organ development. Cells lacking PPB are therefore unlikely to be capable of regenerating a plant. However formation of a preprophase band requires the operation of controls at the molecular level which influence microtubule assembly and disassembly, therefore loss of preprophase bands is probably a secondary consequence of a defect in these controls. Cytoskeletal control is not fully understood in any of the eukaryote kingdoms but is believed to involve microtubule associated proteins (MAPs) (Gotoh et al., 1991; Matsuda et al., 1992) and the activity of protein kinases (Duerr et al., 1993). One important element may be the catalytic activity of p34^{cdc2}. Certainly the kinase reduces microtubule length in extracts of *Xenopus* (Verde et al., 1990) and causes reduction of interphase microtubules when microinjected into mammalian fibroblasts (Lamb et al., 1990). We have observed that the peak of p34^{cdc2}-like protein kinase activity in synchronous cultures of plant cells coincides with the breakdown of preprophase bands and that the coincidence is maintained in cells inhibited with okadaic acid, in which spindle formation and full chromosome condensation do not occur (Chapter 4). Since the establishment of metaphase is not necessary to trigger preprophase band breakdown we have hypothesised (John et al., 1993a) that the trigger may be the activation of p34^{cdc2}, which occurs normally, together with normal preprophase band breakdown, in cells prevented by okadaic acid from reaching metaphase. This possibility is consistent with the observation that a PSTAIR-containing protein like p34^{cdc2} is present in the preprophase band (Mineyuki et al., 1991). A potential element in the loss of preprophase bands from long-term cultures may therefore be high levels of p34^{cdc2}-like kinase.

High levels of p34^{cdc2}-like kinase may also influence the capacity for regeneration by influencing the capacity of cells to exit from the cell cycle and to differentiate. Exit from proliferation is important for organised tissue formation since callus is characterised by the potential division of all cells, whereas in normal plant tissues

cell division is restricted to meristems and cells leaving the meristem are able to enlarge beyond the size that could trigger division. An invariable correlation of low levels of $p34^{cdc2}$-like protein with exit from cell division activity in cells of leaf, stem and root tissue (John et al., 1990; Gorst et al., 1991; reviewed by John et al., 1993a and b) is investigated further and discussed fully in Chapter 6. This correlation is now extended by evidence that, conversely, cells that have levels of $p34^{cdc2}$-like protein and enzyme activity that are four fold and three fold higher than normal are unable to restrain their division and persistently form disorganised callus in which divisions are universally distributed.

We cannot eliminate the possibility that the raised levels of $p34^{cdc2}$-like kinase activity are at least part of the reason for the absence of preprophase bands from the long-term cultured cells. If, as we have hypothesised, $p34^{cdc2}$ kinase activity trigger preprophase band disassembly then a raised basal level of the kinase through the cell cycle could maintain conditions for disassembly. A three fold higher activity of $p34^{cdc2}$-like kinase in samples taken at all times from the synchronous culture (Figs. 5.8 and 5.9) is consistent with a raised basal activity of the kinase.

It is interesting to speculate on the origin of the raised $p34^{cdc2}$-like protein and enzyme activity levels. The simplest possibility is that they arise by random mutations in promoter/enhancer regions of the *cdc2* gene, which results in a higher level of expression that is not selected against during *in vitro* culture. Only when a resumption of controlled division is required in regeneration is the loss of this capacity revealed. An additional possibility is that there is during culture an unintended positive selection for cells with raised $p34^{cdc2}$ levels. It is not clear that raised $p34^{cdc2}$-like protein level must shorten the division cycle of proliferating cells since no direct role in house keeping or growth metabolism has been demonstrated for cdc2. However growth of eukaryotic cells is often slower during S phase and during mitosis, when the genome is occupied in replication or segregation (reviewed by Mitchison, 1971), therefore if raised levels of $p34^{cdc2}$ accelerate these processes the cell cycle may be shorter and individuals with raised levels will have a competitive advantage. Similarly if the fraction of dividing cells present in cell clumps is raised by presence of raised $p34^{cdc2}$ levels then the division

activity of cells with that property will confer a competitive advantage. Since the long-term suspension culture divides rapidly and must be diluted by five sixths every 4 to 7 days a very small proliferative advantage will soon result in a predominance in the population.

In addition to their implications for molecular changes that can occur during prolonged *in vitro*, the data presented in this chapter provide information concerning the possible mechanisms by which levels of $p34^{cdc2}$ catalytic activity are determined during the cell cycle. The observation that levels of $p34^{cdc2}$-like protein can be increased four fold (Fig. 5.7) while a smaller increase in the number of catalytically active molecules is indicated by the three fold increase in enzyme activity (Figs. 5.8 and 5.9) suggests that activity is not limited by levels of the catalytic subunit. Rather the activity may be limited by the levels of cyclins to which it must bind and by the activities of the protein kinases and phosphatase that modify its state of phosphorylation.

A practical application of the new information present here may be that another character can be added to the known requirements for regenerability in a cultured cell line. Gorst et al (1986) have argued that preprophase bands must be present. It can now be suggested that normal low level of $p34^{cdc2}$-like protein must also be present.

Fig. 5.1. Callus from tobacco *N. plumbaginifolia* leaves: cultivated in MS medium containing; (A) 1 mg/l of NAA and 1 mg/l of BAP, (B) 1 mg/l of NAA and 0.4 mg/l of BAP.

Fig. 5.2. Cell suspension culture of tobacco *N. plumbaginifolia* derived from the callus shown in Fig. 5.1.

Fig. 5.1.

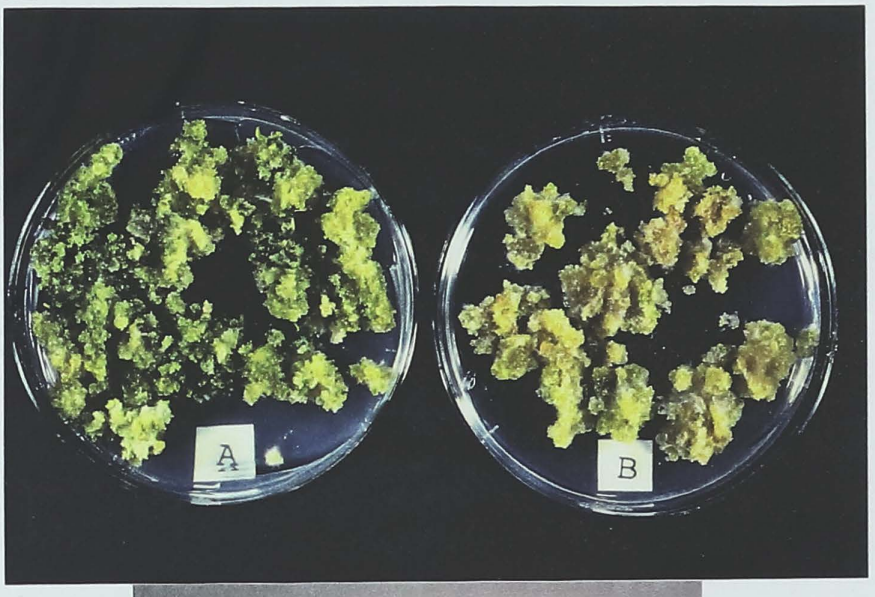


Fig. 5.2.

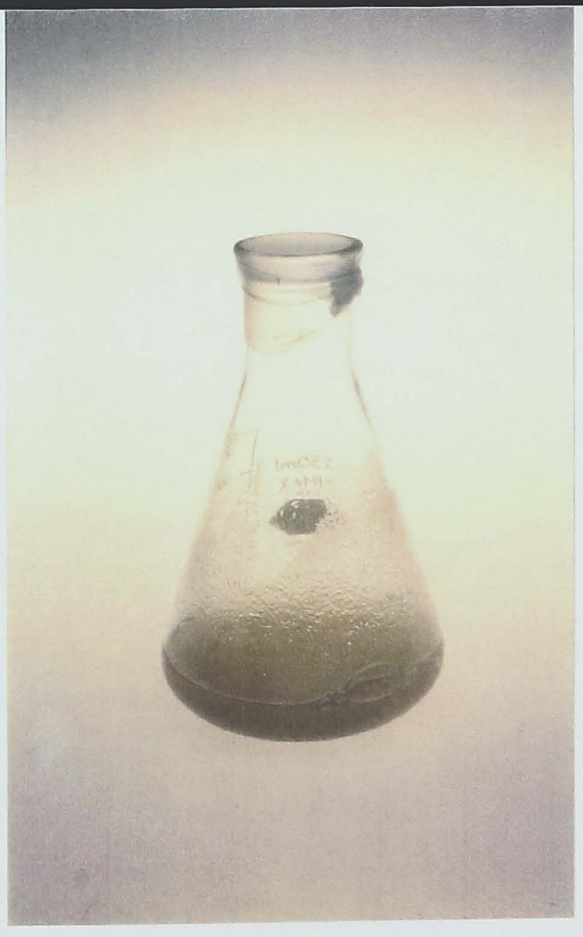


Fig. 5.3. Callus from long-term cell suspension culture of tobacco *N. plumbaginifolia*: cultivated in inducing medium; (A) CSV containing 2 mg/l of 2.4-D and 0.05 mg/l of kinetin; (B) MS medium containing 0.5 mg/l of IAA and 1 mg/l of BAP (B).

Fig. 5.4. Callus having bud differentiation that was derived from the short-term cell suspension culture of tobacco *N. plumbaginifolia*. Callus was cultured in; (A) MS medium containing 0.5 mg/l IAA and 1 mg/l of BAP; (B) MS medium containing 1 mg/l of IAA and 0.1 mg/l of BAP; (C) CSV medium containing 2 mg/l of 2.4-D and 0.05 mg/l of kinetin.

Fig. 5.3.

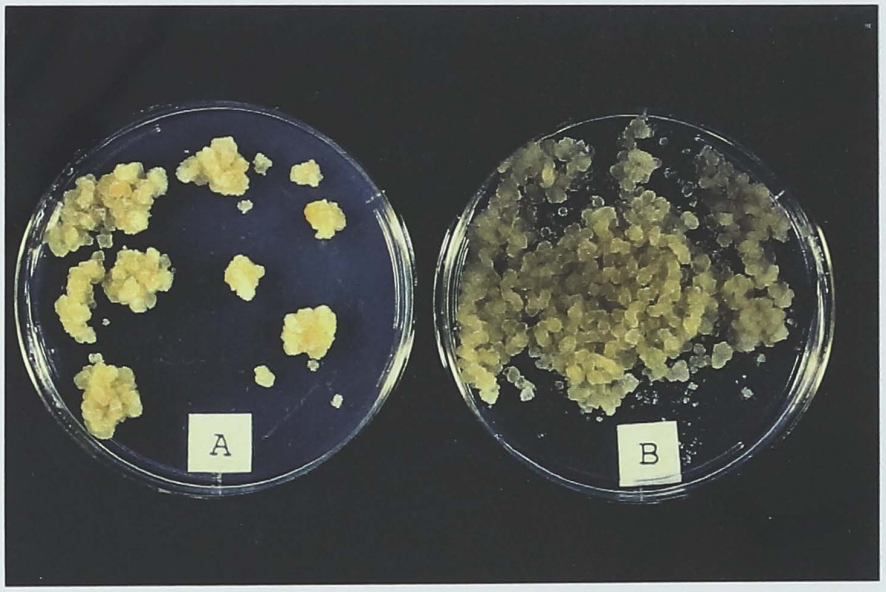


Fig. 5.4.

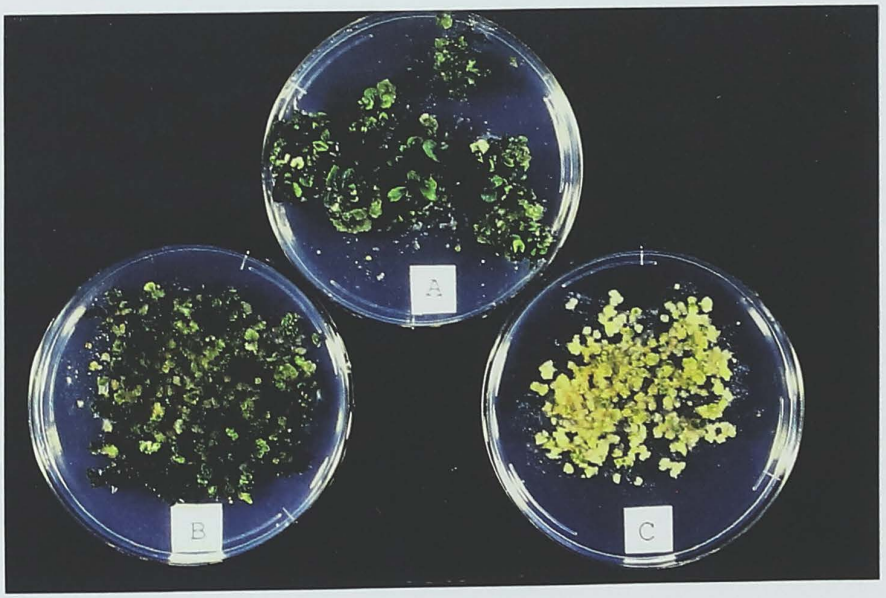
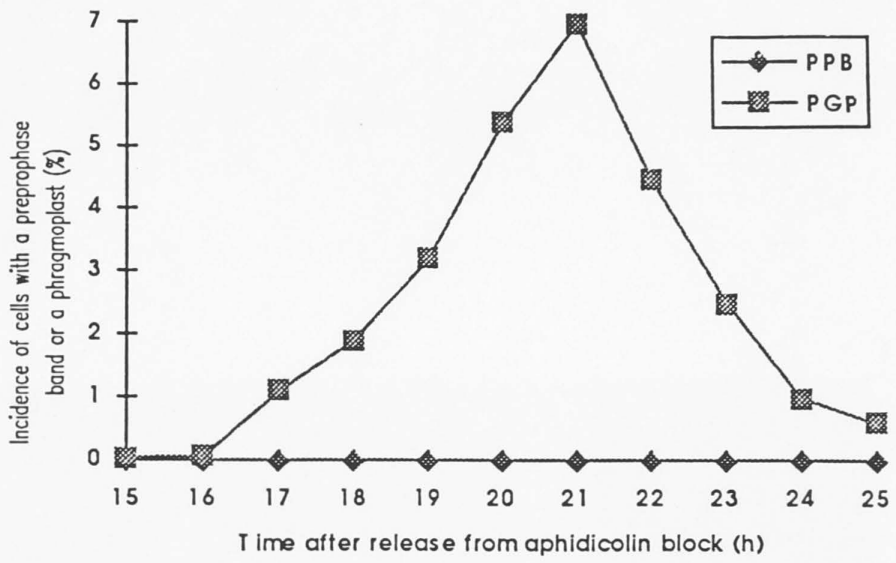


Fig. 5.5. Incidence of cells with preprophase bands of microtubules (PPB) and phragmoplasts (PGP) in; (a) long-term cell suspension culture of tobacco *N. plumbaginifolia* and (b) short term cell suspension culture of tobacco *N. plumbaginifolia*. The cultures were synchronised prior to sampling by treatment with 20 μg/l of aphidicolin for 24h then the inhibitor was removed and samples were taken every 1 hour from 0h to 24h after release from aphidicolin block. Cells were fixed in 4% paraformadehyde and stained with antibody against β-tubulin.

Fig. 5.5.

(a)


Incidence of cells with a preprophase band or a phragmoplast (%)
0
1
2
3
4
5
6
7
15
16
17
18
19
20
21
22
23
24
25
Time after release from aphidicolin block (h)
PPB
PGP

(b)

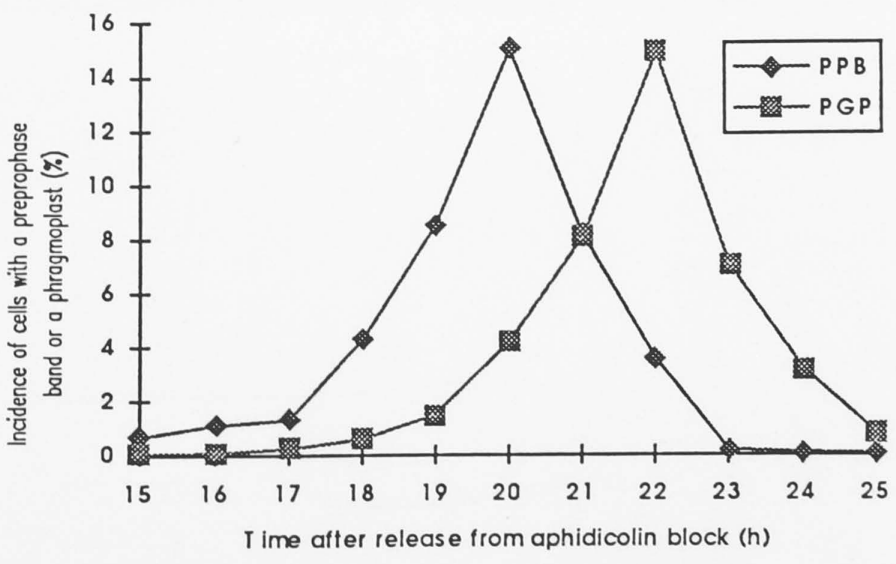

Incidence of cells with a preprophase band or a phragmoplast (%)
0
2
4
6
8
10
12
14
16
15
16
17
18
19
20
21
22
23
24
25
Time after release from aphidicolin block (h)
PPB
PGP

Fig. 5.6. Preprophase bands of microtubules and phragmoplasts detected in short-term cell suspension culture of tobacco *N. plumbaginifolia*. Cells were fixed in 4% paraformadehyde and stained with antibody against β-tubulin. Fig. 5.6.a-g shows the preprophase bands of microtubes and Fig. 5.6.h shows a phragmoplast in this cell suspension culture. Both short-term and long-term cultures formed phragmoplasts but only the short-term culture cells formed preprophase bands. Bar, 5 μm.

Fig. 5.6.

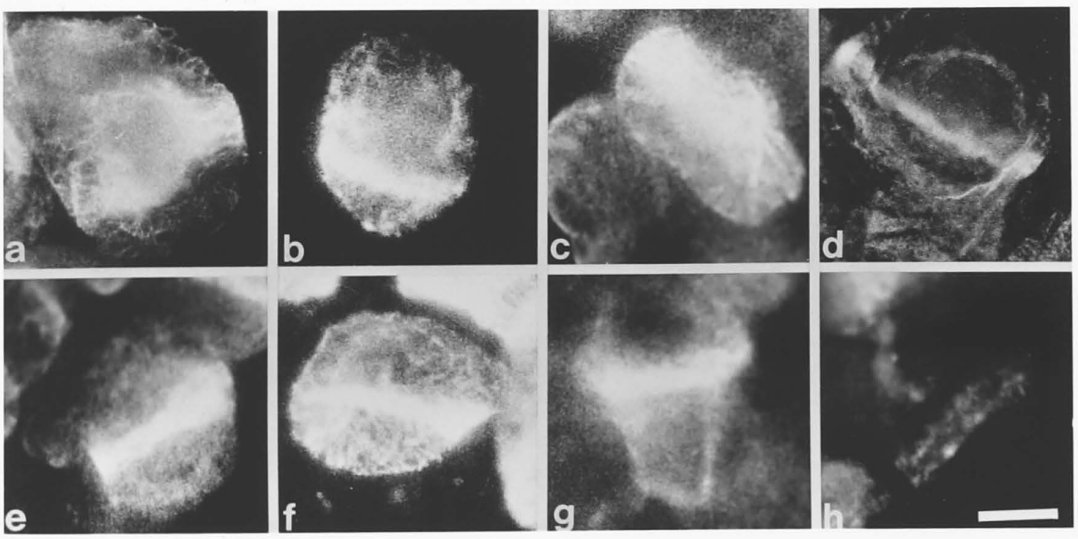
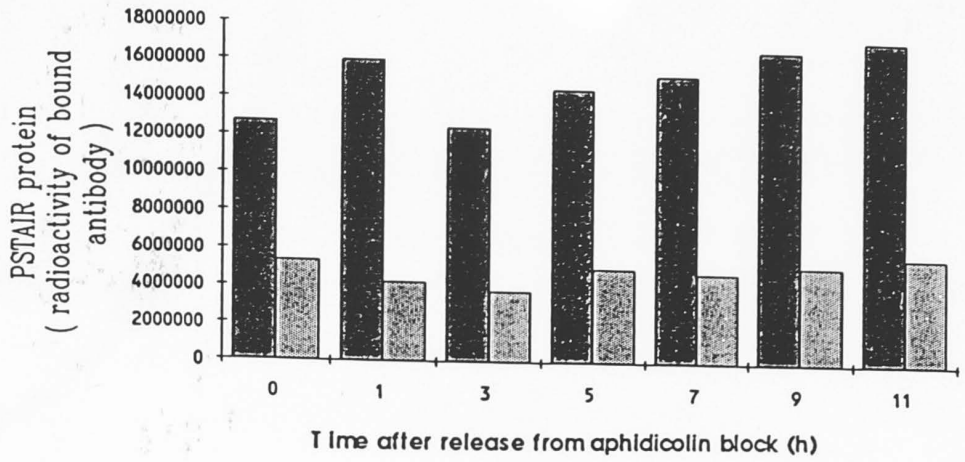
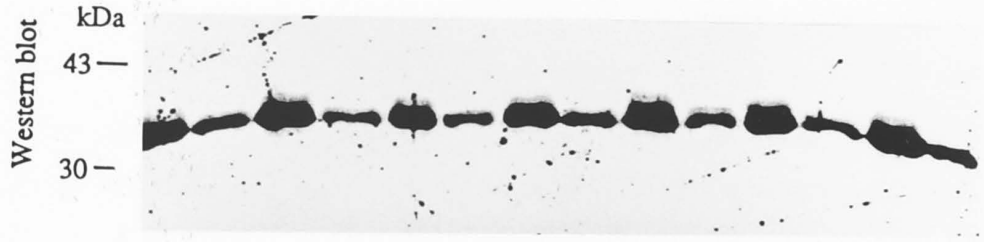
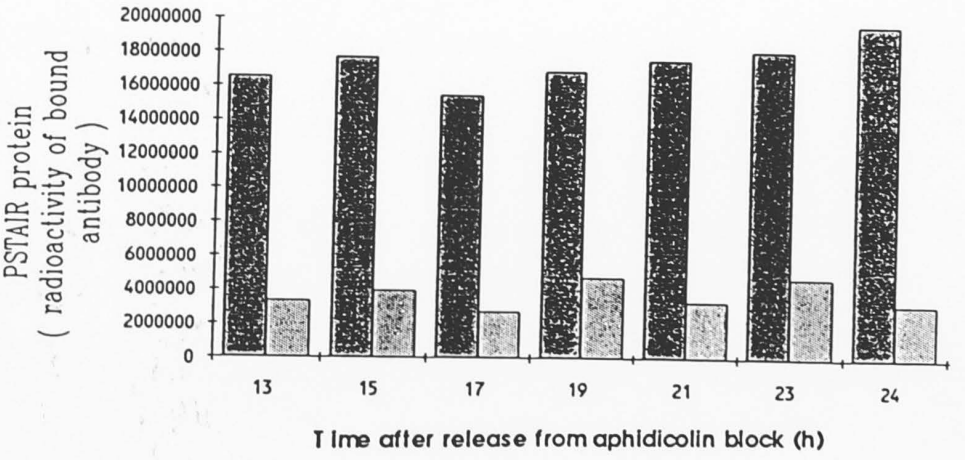


Fig. 5.7. Comparison of levels of $p34^{cdc2}$-like protein in synchronous cultures of long-term and short-term cell suspension culture of tobacco *N. plumbaginifolia*. To allow direct quantitative comparison of the relative amounts of $p34^{cdc2}$-like protein alternate samples from the two types of culture were electrophoresed on the same gel, transferred and probed together. The samples are shown paired and in each case the left hand sample indicates the sample from the long-term culture. Both cultures were synchronised by 24h of arrest with 20 μg/ml aphidicolin then the inhibitor was removed by centrifigation, washing and resuspension of the cells in CSV medium and the sampling period commenced. For each sample total proteins were extracted from 0.1g frozen cell powder in RIPA buffer. Equal loadings of 50 μg protein were separated on 10-15% linear gradient acrylamide gel. Transferred proteins were probed with affinity-purified polyclonal anti-EGVPSTAIRELLKE antibody and bound antibody was detected by ^{125}I anti-rabbit IgG. The image was obtained by exposure in a phopshorImager and analysed to determine levels of $p34^{cdc2}$-like protein by quantification of bound isotope, shown as a histogram above each image.

Fig. 5.7.


PSTAIR protein
(radioactivity of bound
antibody)
18000000
16000000
14000000
12000000
10000000
8000000
6000000
4000000
2000000
0
0
1
3
5
7
9
11
Time after release from aphidicolin block (h)


Western blot
kDa
43
30


PSTAIR protein
(radioactivity of bound
antibody)
20000000
18000000
16000000
14000000
12000000
10000000
8000000
6000000
4000000
2000000
0
13
15
17
19
21
23
24
Time after release from aphidicolin block (h)

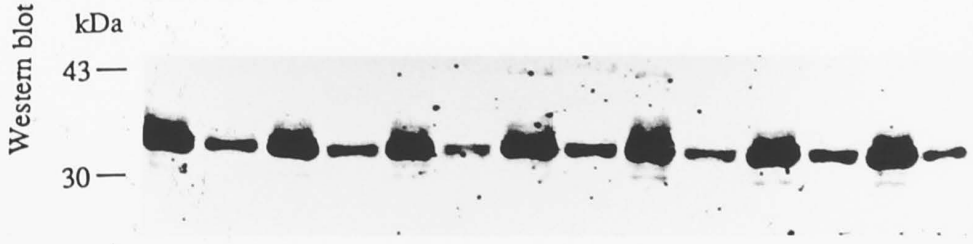

Western blot
kDa
43
30

Fig. 5.8. (a) shows the incidence of DNA synthesis detected by incorporation of BrdU into nuclear DNA, observed in the long-term cell suspension culture that was synchronized with 20 μg/ml of aphidicolin for 24h and assayed for content of $p34^{cdc2}$-like protein (Fig. 5.7). For BrdU labelling, the suspension culture was incubated with 100 μM BrdU for 30 min then probed with antibody against BrdU.

(b) shows the activity of $p34^{cdc2}$-like protein kinase in the synchronised long-term cell suspension culture that was also sampled to determine content of the enzyme protein (Fig. 5.7) and DNA synthesis, (a) above. Also shown are two samples (▲), which were obtained from the equivalent culture (seen in Fig. 5.9) of short-term suspension culture cells at 9h and 21h. These additional samples were extracted, purified and assayed in parallel with the same regents and they therefore provide a rigorous comparison of the enzyme activity in the two cell types. For each sample, $p34^{cdc2}$-like protein kinase was purified from 0.1g of frozen cell powder in NDE buffer by 20 μl of $p13^{suc1}$ beads and eluted with 50 μl of 0.5 mg/ml of $p13^{suc1}$ solution. The activity of $p34^{cdc2}$-like protein kinase was measured using histone H1 as substrate at 30°C for 5 min. The amount of ^{32}P transferred was measured by placing 20 μl of reaction mixture on P81 phosphocellulose paper washing in phosphoric acid and counting in a scintillation counter. PhosphorImaged histone H1 was obtained by separating 30 μl of reaction mixture on 12% acrylamide gel and exposure in a phosphorImager.

Fig. 5.8.

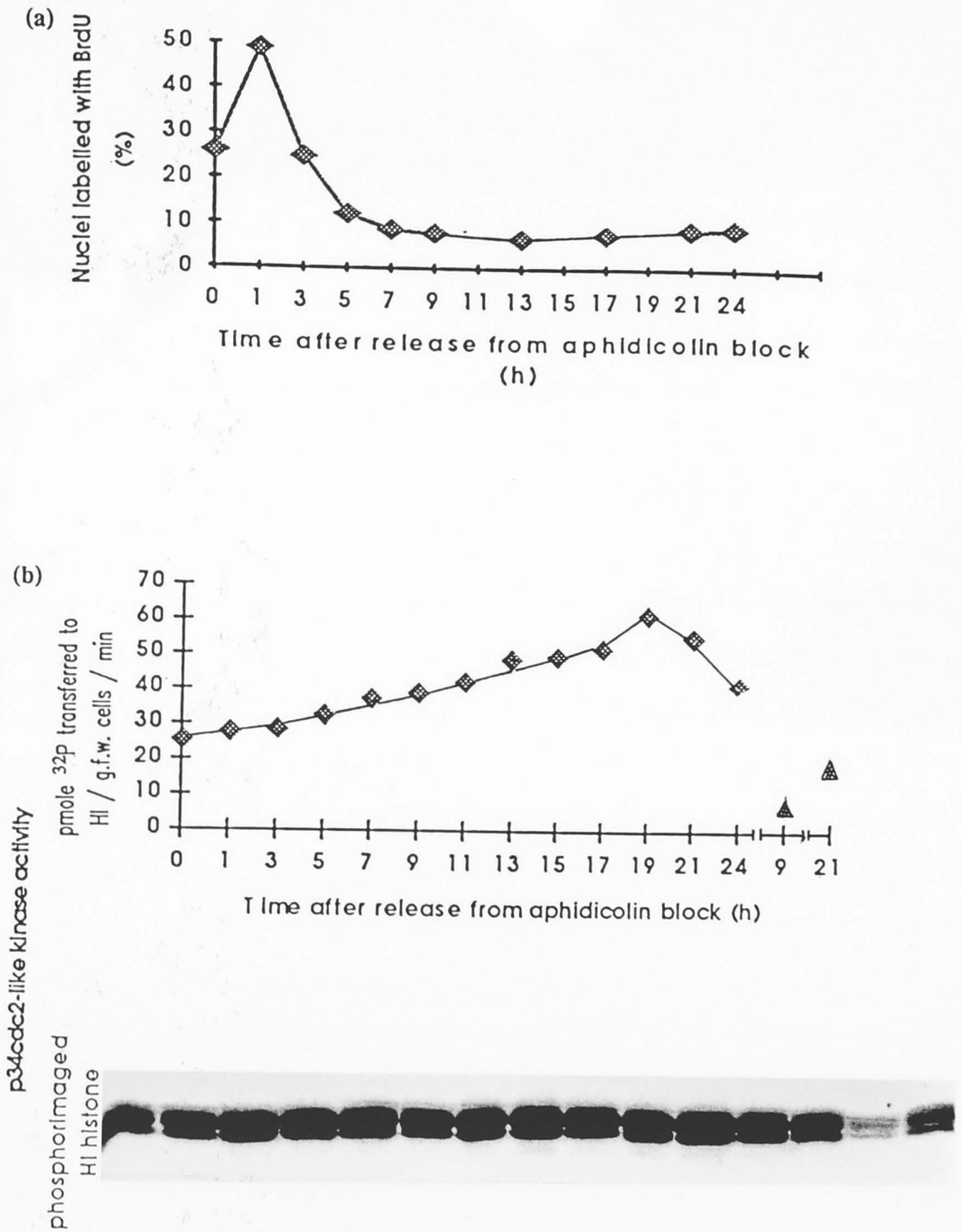
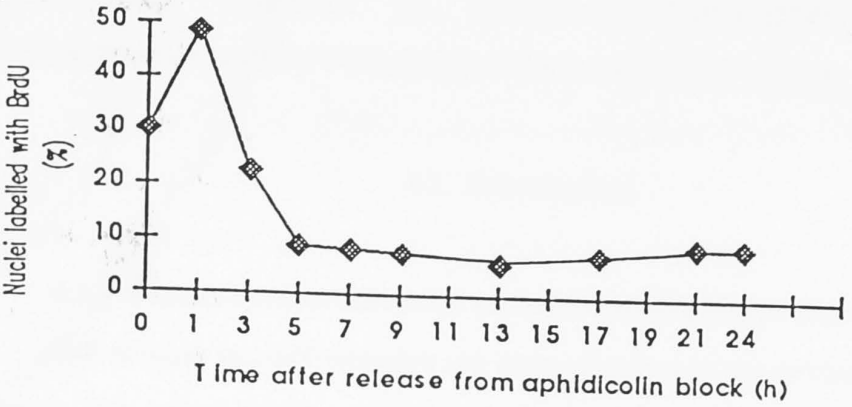

(a)
Nuclei labelled with BrdU (%)
50
40
30
20
10
0
0 1 3 5 7 9 11 13 15 17 19 21 24
Time after release from aphidicolin block (h)
(b)
p34cdc2-like kinase activity
pmole 32p transferred to HI / g.f.w. cells / min
70
60
50
40
30
20
10
0
0 1 3 5 7 9 11 13 15 17 19 21 24 9 21
Time after release from aphidicolin block (h)
phosphorImaged HI histone

Fig. 5.9. (a) shows the incidence of DNA synthesis detected by incorporation of BrdU into nuclear DNA, observed in the short-term cell suspension culture that was synchronized with 20 μg/ml of aphidicolin for 24h and assayed for content of $p34^{cdc2}$-like protein (Fig. 5.7). For BrdU labelling, the suspension culture was incubated with 100 μM BrdU for 30 min then probed with antibody against BrdU.

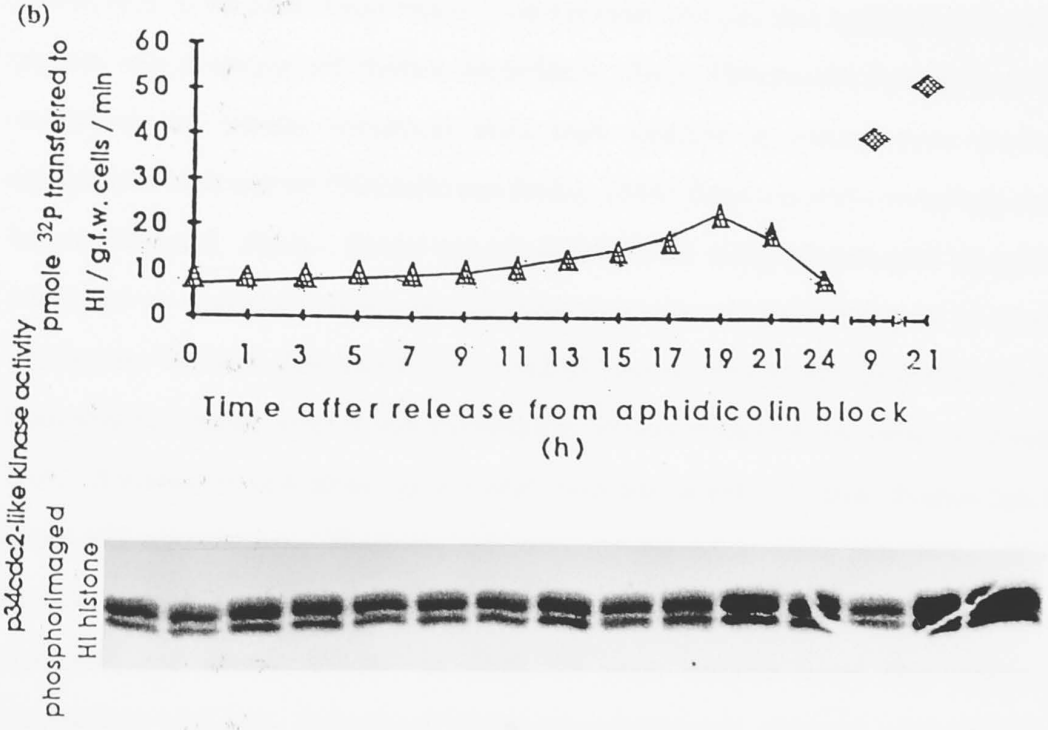
(b) shows the activity of $p34^{cdc2}$-like protein kinase in the synchronised short-term cell suspension culture that was also sampled to determine content of the enzyme protein (Fig. 5.7) and DNA synthesis, (a) above. Also shown are two samples (▦), which were obtained from the equivalent culture (seen in Fig. 5.8) of long-term suspension culture cells at 9h and 21h. These additional samples were extracted, purified and assayed in parallel with the same regents and they therefore provide a rigorous comparison of the enzyme activity in the two cell types. For each sample, $p34^{cdc2}$-like protein kinase was purified from 0.1g of frozen cell powder in NDE buffer by 20 μl of $p13^{suc1}$ beads and eluted with 50 μl of 0.5 mg/ml of $p13^{suc1}$ solution. The activity of $p34^{cdc2}$-like protein kinase was measured using histone H1 as substrate at 30°C for 5 min. The amount of ^{32}P transferred was measured by placing 20 μl of reaction mixture on P81 phosphocellulose paper washing in phosphoric acid and counting in a scintillation counter. PhosphorImaged histone H1 was obtained by separating 30 μl of reaction mixture on 12% acrylamide gel and exposure in a phosphorImager.

Fig. 5.9.

(a)


Nuclei labelled with BrdU (%)
50
40
30
20
10
0
0 1 3 5 7 9 11 13 15 17 19 21 24
Time after release from aphidicolin block (h)

(b)


pmole 32P transferred to HI / g.f.w. cells / min
60
50
40
30
20
10
0
0 1 3 5 7 9 11 13 15 17 19 21 24 9 21
Time after release from aphidicolin block (h)
p34cdc2-like kinase activity
phosphorimaged HI histone

CHAPTER 6

CHANGES IN THE PROTEIN LEVEL AND CATALYTIC ACTIVITY OF $p34^{cdc2}$-LIKE ENZYME IN CYCLING, DIFFERENTIATING AND DEDIFFERENTIATING TOBACCO CELLS AND PITH TISSUE

6.1 Introduction

6.1.1 Phytohormonal influences on cell division in higher plants

Cell division and differentiation are basic processes in the development of higher plants. The notion that the initiation of cell division in higher plants is induced by endogenous growth substances can be traced back a 100 years to Wiesner, and especially to Haberlandt who, in the early years of this century, found that a substance that could be derived from phloem tissue and was diffusible through a thin film of agar could induce cell division in the parenchyma tissue of potato tuber and also that application of crushed phloem cells promotes cell division (Haberlandt, 1921). Haberlandt's findings have been confirmed and similiar substances have been detected in various plant products, especially in malt extract (Blakeslee and Satina, 1944), *Datura* embryo extract and carrot leaves (Wiggans, 1954). Skoog and his collaborators using tobacco pith as material attempted to attribute specific cell division responses to specific classes of growth substances. In 1954, they found that no cell division ocurred in pith tissue that was free from vascular tissue, when it was cultivated in White's medium in the absence of added IAA. Addition of IAA alone, to 2-3 mg/l, was also unable to cause division but did cause cell enlargement. However, cell divisions did occur when pith with attached vascular strands or in contact with vascular tissue was cultivated in White's medium (Jablonski and Skoog, 1954). In 1956, the same workers found that kinetin, in combination with IAA, induced cell division in tobacco pith, although neither IAA nor kinetin alone could induce division (Das et al., 1956). The dual requirement for both kinetin and auxin to induce division in tobacco pith has been confirmed (Bottomley et al., 1962; Simard, 1971).

Up to the mid 1960's, cytokinin-containing extracts that could combine with auxin to induce cell division in pith had been prepared from approximately forty species of higher plants, especially coconut milk and maize kernals but also developing fruits of apple, quince, pear, plum, peach and tomato (Bottomley et al., 1962; Letham and Williams, 1969; Powell and Pratt, 1964), and in germinating lettuce (Barzilai and Mayer, 1964), barley (Srivastava, 1963) and pea (Zwar and Skoog, 1963) seeds. The natural cytokinin zeatin, which can induce cell division at 0.1μg/l *in vitro*, was purified and identified in the 1960's (Letham, 1966). Addition of both cytokinins and auxins can influence the occurence of cell division in many higher plant tissues.

In higher plants, cell division occurs in meristems from which all plant organs are ultimately derived. Unlike a healthy human embryo which has all its limbs formed prior to birth so that post-natal development is essentially enlargement of an existing pattern, a germinating plant embryo will initiate a new pattern of development throughout its life cycle. So the basic function of meristems is to continuously to produce new cells by cell division and to produce specific organs and tissues by differentiation of these cells. Light, gravity and phytohormones can influence cell division and differentiation in the meristems in higher plants. For example, a low concentration (5×10^{-8}M) of kinetin stimulated the initiation of pea lateral roots, whereas at higher concentration, kinetin was inhibitory in root meristems (Torrey, 1962). Cytokinin also influences tissue differentiation and xylem development in radish roots (Loomis and Torrey, 1964). Both auxin and cytokinin in increasing amounts could alter the site of lateral root formation in *Convolvulus* root, and gradients of auxin and cytokinin along the longitudinal axis of roots are probably of great significance in regulating the initiation of lateral roots in root meristems (Bonnett and Torrey, 1965; Wightman and Thimann, 1980; Wightman et al., 1980).

6.1.2 Functions of cytokinin in cell division and differentiation in higher plants

The study of how plant growth substances may function in the regulation of cell division in higher plants has been underway for more than 30 years. Some studies have used what may be supraoptimal concentrations of hormone. Van't Hof (1968) found that when excised pea root apical meristems were made quiescent by sucrose starvation and then stimulated with sucrose the presence of 1 x 10^{-7} M IAA and 3.2 x 10^{-6} M kinetin was inhibitory to resumption of division, although when applied to whole roots at the same concentration they stimulated lateral root formation. The presence of IAA and kinetin reduced the initiation of S phase and also of mitosis and the presence of IAA prolonged S phase. Similarly Levi et al (1987) found that BAP applied to intact seeding pea roots at 30 μM inhibited progress from G1 to S phase and from G2 to M phase. The *in vivo* significance of these effects of supraoptimal external hormone concentrations is uncertain. They could indicate that cell division activity could sometimes occur by a reduction of hormone levels below levels that were previously inhibitory.

The converse possibility, that division might arise by the elevation of hormone levels that were previously suboptimal, is indicated by the effects of hormones on excised tissues. There is a considerable amount of evidence showing that cytokinin alone cannot induce division but that it must be present together with auxin for cells to progress through G2 phase, mitosis and cytokinesis. However the precise cycle event(s) that are cytokinin dependent have not previously been identified. For example, an early conclusion from Skoog's laboratory was that cytokinin might be necessary for the process of cytokinesis itself and this presumably influenced the naming of the class of hormone (Naylor et al., 1954). The classic work of Skoog's group on tobacco pith showed that tissue lacking cytokinin could synthesize DNA but not increase in cell number; therefore providing an early indication that progress from G2 through mitosis is dependent at least at some point upon cytokinin, however it was not demonstrated that cells lacking cytokinin were arrested in G2 phase. Jouanneau and Tandeau (1973) found that in tobacco cell suspension culture auxin could sustain DNA synthesis when present alone but further progress through the cell cycle then required the presence of cytokinin.

In tobacco cultures resuming division after protoplasting of mesophyll cells, the presence of auxin is necessary from the beginning of culture, while cytokinin is only required later to allow development of the mitotic apparatus (Meyer and Cooke, 1979). However Bayliss (1985) concluded that it is unclear whether there is a true hormone dependent G2 control point as most studies have failed to properly demonstrate G2 arrest. Bayliss (1985) suggests that cytokinin involvement at the putative G2 control point may be concerned with the switch to endoreduplication cell cycles.

These studies leave open the question of what specific cell cycle events at the molecular level are stimulated by the presence of cytokinin. Cytokinin markedly promotes protein synthesis during the cell cycle by inducing polyribosome formation in cell suspension cultures of *Glycine max* (L) Merr (Fosket et al., 1977) and by enhancing the specific activity of the polysomal-mRNAase (Tepfer and Fosket, 1978). It was further demonstrated that the cytokinin-induced synchronous mitotic wave in tobacco cell suspension was effectively blocked by the protein synthesis inhibitor 5-methyltryptophan (Jouanneau, 1975), but the mitotic activity in cultured cells of *Glycine max* was not affected by the RNA synthesis inhibitors actinomycin D or 5-fluorouridine (Tepfer and Fosket, 1978). Thus, it was proposed that cytokinin-induced cell division is regulated at the translational level, possibly *via* the synthesis of a specific protein. However, such a claim is premature since there is evidence from some cell types that cytokinin is involved in DNA as well as RNA synthesis in the cell cycle. In *Petunia* protoplast cultures, which require both cytokinin and auxin for cell division (Cocking et al., 1977; Bergounioux-Bunisset and Perennes, 1980), it was found that cytokinin alone can induce the highest RNA level in the G1 phase although both cytokinin and auxin are essential for entry into the S phase. While in the S phase, there is a clear dependence of RNA synthesis upon cytokinin, then both hormones were required for attainment of highest RNA levels in G2. Hence, it was suggested that the primary action of both cytokinin and auxin leading to mitosis appears to be in the S phase (Bergounioux et al., 1988).

One possibility is that cells in different tissues have different hormone requirements or sensitivities at the same cell cycle points.

6.1.3 Functions of auxin in cell division, growth and differentiation in higher plants

Auxin plays an important role in higher plant developmental processes, especially in cell division and cell elongation. Auxin and cytokinin together induce tobacco pith and suspension culture cells to divide and cells cannot enter into the S phase when auxin is lacking (Jouanneau and Marsac, 1973; Fraser, 1975 a, b; Cook and Meyer, 1981). Wheat leaf meristematic explants continued cell division in medium containing auxin, but in the absence of auxin they soon ceased dividing and were arrested in both G1 and G2 phase (Wernicke and Milkovits, 1987). This evidence suggests that auxin is required for cell division and growth and that explants are frequently dependent on exogenous auxin.

There is considerable evidence that auxin regulates the expression of genes in plant cells and organs. Some research has been aimed at discovering which genes are influenced by auxin but their function in establishing or catalyzing division is unknown. In protoplasts from tobacco mesophyll cells Takahashi et al (1989) detected three genes that were expressed within 15 minutes of auxin treatment and responded specifically to auxin since other plant hormones, such as gibberellic acid, kinetin, abscisic acid and ethylene were unable to cause or interfere with the IAA-induced mRNA accumulation. Expression was also unaffected by anaerobic, heat or cold stress. Expression of these genes in pea tissue ocurred concomitantly with the initiation of cell elongation by auxin (Theologist et al., 1985). Some of the auxin-regulated genes from soybean were cloned, sequenced and characterized (Hagen et al., 1988; Ainley et al., 1988; McClure et al., 1989; Guilfoyle, 1990; 1991). The regulation of auxin-responsive genes is tissue-specific and organ-specific, and time-course experiments showed that the auxin-inducible polypeptides were detected within 30 minutes of hormone application and reached a constant level after 2h to 4h, on the other hand the two proteins whose levels were reduced by auxin were affected after 6 hours (Gee et al., 1991). It has been postulated that proteins whose synthesis is stimulated in the presence of auxin are possible candidates for a role in the induction of the cell cycle whereas proteins reduced by auxin are involved in cell wall formation (Meyer and Chartier, 1981).

The above observations indicate that there is still no evidence to clearly show how cytokinin and auxin regulate the gene (s) or their product that may control division activity. It is therefore timely to investigate the effects of phytohormones on the accumulation and activity of $p34^{cdc2}$, which has an essential and well characterized role in eukaryote cell cycles.

6.1.4 Involvement of $p34^{cdc2}$-like protein in dividing, differentiating and dedifferentiating cells of wheat and of carrot

Changes in level and enzyme activity of $p34^{cdc2}$-like protein are consistent with a role in the regulation of division, differentiation and dedifferentiation in cells of wheat leaf and carrot cotyledon. Seedling wheat leaves have a linear developmental gradient from the meristematic region at the base through progressive stages of differentiation to mature photosynthetic cells. Mitotic activity is restricted to the lower 8 mm and coincides with maximum levels of $p34^{cdc2}$-like protein detected by PSTAIR antibody (John et al., 1990) and also coincides with high activity of $p34^{cdc2}$-like protein kinase using histone H1 as substrate (John et al., 1993b). A decline of $p34^{cdc2}$-like protein relative to other proteins correlates with a switch to cell differentiation and the basal level of $p34^{cdc2}$-like protein in differentiated cells is one-sixteenth of that in dividing cells. When segments from the meristematic region of wheat leaves are cultured on agar medium, auxin (2,4-D) is able to stimulate continued synthesis of $p34^{cdc2}$-like protein and cell division, resulting in callus from which shoots and roots can differentiate. However the capacity of the same medium to stimulate synthesis of $p34^{cdc2}$-like protein and division in cells outside the meristem declines roughly in proportion with the level of $p34^{cdc2}$-like protein (John et al., 1990) and more precisely in proportion with the level of $p34^{cdc2}$-like protein kinase activity, which is more sharply restricted to the meristem (John et al ., 1993a).

Cells in dictoyledonous plants are more readily stimulated to resume division. The carrot cotyledon completes cell division in the first two days after germination and then enters a period of more than tenfold cell enlargement and differentiation to form photosynthetic tissue. A more than tenfold decline in $p34^{cdc2}$-like protein coincided with

cessation of division. When the segments of 11 day-old cotyledons were transferred to nutrient agar containing 2,4-D an induction of $p34^{cdc2}$-like protein with no discernable lag preceded dedifferentiation and resumption of division, beginning in cells adjacent to the vascular tissue that may have been first to receive the hormone. The spread of division activity through the tissue correlated with the steady increase of $p34^{cdc2}$-like protein (Gorst et al. 1991). In root tissue also the induction of $p34^{cdc2}$-like protein precedes resumption of division and the speed with which auxin induces increase in cdc2 mRNA, detectable by 10 minutes, indicates a possible direct effect on the *cdc2* gene (John et al., 1993b). These results suggest that decline in $p34^{cdc2}$-like protein may be involved in switching off division prior to differentiation and that restoration of its levels conversely required for switching on the resumption of division in differentiated cells that are induced by phytohormone.

6.1.5 Unresolved problems

If we accept the possibility that the cell division cycle of some, perhaps all higher plants needs the presence of both auxin and cytokinin and the possibility in plant cells, as in yeasts, that $p34^{cdc2}$ protein kinase controls the transition from late G1 to S phase and the transition between late G2 phase and M phase (reviewed by Norbury and Nurse, 1992) then three questions arise. The first is, to what extent do phytohormones that influence the switch between cessation and resumption of division act upon *cdc2* gene expression and what is the mechanism if this ocurs? Secondly, what are the relationships between auxin, cytokinin and possible changes in catalytic activity of $p34^{cdc2}$ protein. Thirdly, can dependence upon specific phytohormones regulate progress through specific phases of the cell cycle? perhaps by influencing the activation of $p34^{cdc2}$ kinase at key cell cycle transitions. In this Chapter, I report experiments that used cell suspension culture of *N. plumbaginifolia* to study (1) the relation between exogenous phytohormones and the synthesis of $p34^{cdc2}$-like protein, (2) whether auxin and cytokinin-dependent transitions can be detected in their cell cycle, and (3) the relationship between exogenous auxin and cytokinin and activity of $p34^{cdc2}$-like protein kinase. A parallel set of experiments used tobacco pith (Wisconsin 38) to study the

dedifferentiation induced by auxin and cytokinin, and the relationship between the de-differentiation and the change of content and of kinase activity of $p34^{cdc2}$-like protein.

6.2 Materials and Methods

6.2.1 Arrest and resumption of division in cell suspension cultures of *N. plumbaginifolia* by omission and resupply of auxin and cytokinin

Cell suspension cultures of *N. plumbaginifolia* were usually diluted every four days in standard CSV medium. To investigate hormone dependence cells scheduled for dilution were harvested by spinning down at 3500 rpm in a bench (Clements 2000) centrifuge for 2 min, rinsed twice by centrifugation in hormone free CSV medium (CSV 0), resuspended in this medium to the standard initial culture density of 1 x 10^6 cells per ml and cultured for four days. During this 4 day period some residual cell division ocurred. After 4 days culture in medium without added hormone the suspension was again diluted to one sixth in the same hormone free medium, but without centrifugation. In this second four day period residual carried-over hormone was more thoroughly depleted. The arrested cells were then ready for restimulation by 2,4-D and/or kinetin.

The 8-day hormone depletion treatment was employed in two types of experiment that monitored resumption of division on resupply of hormones. An early type of experiment (Fig. 6.1) was designed to test whether cells had been made non-viable by lack of hormone in which case negative responses to attempted restimulation might be misleading. For this test the 8-day-depleted cells were treated for a further 4 days during sampling in the following hormone mixture; (1) no hormone; (2) kinetin only; (3) auxin only; (4) auxin and kinetin. Then to test viability and capacity to resume proliferation the cultures were supplemented so that all contained auxin and kinetin and were sampled for a further 4 days (Fig. 6.1). Cells that did not recieve both hormones until the final 4 day period (treatment 1), and therefore had been deprived for 12 days previously, responded almost as promptly with resumption of division as did cells that were provided both

hormone after 8 days (Fig. 6.1. treatment 4). Therefore 12 days of hormone depletion did not significantly reduce cell viability and in all subsquent experiments this depletion period did not exceed 8 days, consequently loss of viability is not a complicating factor in these experiments.

Later experiments (shown in Figs. 6.2-6.9) retained the 8 day hormone depletion treatment but used only a 2 day treatment with different hormone mixtures, followed by a 42h period of supplementation with hormones in test cultures that were sampled. In these experiments 2,4-D was always supplied at 9 μM and kinetin was supplied at 0.23 μ M.

Samples taken during arrest and resumption of cell division and growth were analysed for nuclear DNA content, cell number density, the levels of $p34^{cdc2}$-like protein and the protein kinase enzyme activity of $p34^{cdc2}$-like protein.

6.2.2 Induction of dedifferentiation in stem pith tissue of *N. tabacum* cv Wisconsin Havana 38 by auxin (NAA) and cytokinin (BAP)

6.2.2.1 Preparation of the stem pith tissue of *N. tabacum*

Plants of *N. tabacum* cv Wisconsin Havana 38 were grown in soil in a glasshouse maintained at 30°C during a 10h day and at 25°C by night. High mineral nutrition and high light intensities were provided and the plants were cultivated for about four months. Sections of stem about 60 cm long were harvested and segments 15 cm long were cut from these and surface sterilised by immersion for 20 min in 20% sodium hypochlorite then rinsed twice in sterile water. All xylem and internal phloem were cut off the sterilised stem pieces and then 10 mm long cylindrical segments of central pith tissue were cut. Each segment was split longitudinally into four quadrant blocks which were dispersed to sterile solid MS media with four different combinations of NAA and BAP.

6.2.2.2 Media and culture conditions for excised tobacco pith

MS media with four different combinations of auxin and cytokinin were used to test for induction of dedifferentiation in pith blocks; (1) MS 0, NAA free and BAP free;

(2) NAA-only, containing 5.4 μM of NAA (1 mg/l); (3) BAP-only, containing 0.56 μM of BAP (0.2 mg/l) and (4) complete MS, containing 5.4 μM of NAA and 0.56 μM of BAP. Pith blocks were incubated at 25^{0}C with 8h light and 16h darkness. Samples for detection of nuclear DNA synthesis and measurements of the protein levels and enzyme activity of p34^{cdc2}-like protein kinase were taken at 8, 14 and 20 days.

6.2.3 Detection of nuclear DNA synthesis in tobacco pith tissue

6.2.3.1 Labelling pith callus with BrdU

Samples of pith callus, which had been cultivated on MS 0, BAP-only, NAA-only or complete MS medium for 8, 14 and 20 days, were cut into smaller pieces, each 5 x 2 x 2 mm, and these were incubated in the liquid MS of the same hormone content as the solid medium from which they were taken and in each case containing 100 μM BrdU and 3% (v/v) hydrogen peroxide. Incubation was for 15h at 25^{0}C with 100 rpm shaking then the tissue was fixed in 4% (v/v) parafomaldehyde in PBS for 1h and infiltrated, in PBS containing 0.4% (v/v) paraformaldehyde and 1 M of sucrose, for 3h, then rinsed twice with 1.3 M sucrose and infiltrated with 1.3 M sucrose until the tissue settled to the bottom of the solution. The samples were then ready for frozen sectioning.

6.2.3.2 Frozen sectioning of pith callus

The samples for sectioning were placed in liquid tissue-Tek (Miles Inc., Elkhart, IN) in a plastic mold (cryomold, Miles Inc.), and plunged into liquid nitrogen. The frozen block was then stored at -20^{0}C before sectioning. Sections were performed on a Reichert cryotome (2800 Frigocut E) set to cut 8 μm sections. Sections were readily transferred onto a breath-moistened poly-lysine-coated glass slide placed just above the ribbon. Sections were left to settle onto the slide for at least 15 minutes.

6.2.3.3 Immunofluorescence detection

The sections, after rinsing 2 x 3 minutes in PM5E buffer (50 mM PIPES, 2mM $MgSO_4$, 5mM EGTA and pH 6.8 adjusted with NaOH), were digested in 2% (w/v)

cellulysin cellulase (Calbiochem 219466) in 0.4 M Mannitol PM5E buffer for 10 minutes at room temperature. After rinsing in PM5E buffer 2 x 3 minutes cells were permeabilised in 1% NP40 in PM5E buffer for 15 minutes. After rinsing off NP40, the sections were incubated in 20-30 µl of anti-BrdU antibody (Amersham) at 37°C for 3h or overnight. After rinsing off the first antibody, the sections were incubated for 1-2h with an FITC-conjugated sheep anti-mouse IgG antibody (SAM: Silenus Lab Ltd., Dandenong, Australia) diluted 1:30 in PBS with 1% (w/v) bovine serum albumin and 0.02% (w/v) NaN3 at 37°C. After rinsing off the secondary antibody by 3 washes with PBS the preparations were also stained with 0.2 µg/ml DAP1 in PBS for 3-5 minutes at room temperature to label DNA. Sections were mounted in glycerol-polyvinyl alcohol (Mowiol; Hoechst A.G., Frankfurt, FRG) containing 0.1% paraphenylenediamine.

Nuclei labelled with BrdU-associated fluorescence were detected by use of Zeiss Axioplan and Axiovert microscopes equipped with epifluorescence optics at 400-500 nm light.

6.3 Results

6.3.1 Effects of 2,4-D and kinetin on cell proliferation in suspension cultures of *N. plumbaginifolia* cells

The suspension culture of *N. plumbaginifolia,* that was investigated in the experiments described in this Chapter has been cultured *in vitro* for more than five years, and its cells now divide very rapidly. In order to investigate its dependence upon exogenously supplied 2,4-D and kinetin the suspension culture was deprived of added hormones then the effects of re-addition of hormones was studied. The culture was routinely propagated by standard dilutions every four days from a cell density of about 6 x 10^6 per ml to one sixth of that density. To investigate hormone dependence cells were spun down and washed twice in hormone free CSV medium (CSV 0) then resuspended in CSV 0 medium and cultivated for four days during which time division activity ceased. They were then diluted in CSV 0 again to one sixth of cell number density, and incubation was continued for a further four days during which no division occurred.

After this 8 day treatment the cells had completely stopped dividing. This was evident from their behaviour when sampled in the next 4 day period in medium that continued to lack both auxin and cytokinin (curve 1, 0 to 4 days in Fig. 6.1). The cells were however viable, although quiescent in term of proliferation, because when supplemented (arrow in Fig. 6.1) at day 4 in the sampling period with 2,4-D at 9 μM and kinetin at 0.23 μM the cells grew and divided, increasing in number from 8×10^3 to 1.6×10^4 per ml.

A requirement for both auxin and cytokinin was indicated in cells that had been depleted for 8 days, since in the first 4 days of supplementation (days 0-4 of the sampling period, Fig. 6.1) neither 2,4-D alone at 9 μM (curve 3) nor kinetin alone at 0.23 μM (curve 2) could support proliferation, although cells provided with both (curve 4) responded by increasing in number from 8×10^3 to 2.5×10^4 per ml.

The appearence of the cultures after incubation in the partial hormone mixtures is shown in Fig. 12. Cultures lacking auxin developed a brown colouration that may signify a switch to production of secondary metabolites. A similar colouration developed in tobacco pith under equivalent partial hormone treatment (Fig. 6.13). In the case of the suspension cultures it is clear that the cells remained viable since they could resume cycling on resupply of both auxin and cytokin (days 4 to 8 Fig. 6.1).

It is concluded that the division and growth of this cell suspension culture of *N. plumbaginifolia* depends on the supply of both auxin and cytokinin in the culture medium.

6.3.2 Effects of 2,4-D and of kinetin on the DNA content of *N. plumbaginifolia* cells in suspension culture

Nuclear DNA contents of most of the cells that were cultured in CSV 0 medium for 10 days and could not divide and grow (Fig. 6.2.b), are characteristic of G1 phase but the skewing towards higher values suggests that some cells may have been in G2 or possibly S phase. Arrest in S phase is not usual in eukaryote cells and therefore this conclusion is drawn only tentatively. Culture for 2 days in kinetin-only medium, after 8 days of hormone depletion, resulted in a mixed population of cells some of which were in G1 phase, indicated by a peak at 60 units of DNA, and others in G2, peaking at 100 units

(Fig. 6.4.b). This profile indicates that the lack of auxin restrained progress both from G1 to S and from G2 to M phases resulting in the persistence of cells in both G1 and G2 phases. It is consistent with the deduced arrest in both G1 and G2 phases by fractions of the same population that two periods of cell number increase were detected when the missing auxin was supplied (Fig. 6.4.a). An increase in the first 12h presumably represented division by cells previously arrested in G2 phase and the later increase in cell number beginning at 24h presumably derived from cells previously arrested in G1 phase that had progressed during the sampling period through a whole cell cycle. By contrast, cells cultured for 2 days in auxin-only medium were able during that time to progress through G1 and S phases and accumulate in G2 phase since the nuclear DNA content (Fig. 6.6.b) shows a single peak around 110 units. Subsequent cell number increase confirms the deduced arrest since the whole population divided when missing cytokinin was supplied and cell number doubled in a single wave of division in the first 18h (Fig. 6.6.a). Control cells that were cultured for 2 days in medium with both auxin and cytokinin after the 8 day depletion divided once. This is reflected in a cell number at the end of the 2 days (which is the beginning of the 42h sampling period) that was 12×10^4 and therefore about double that in the zero or partially supplemented cultures at the equivalent time. Within the 2 day period the cells had entered the next cell cycle and reached G2 phase as indicated by their nuclear DNA content that was predominatly 110 units (Fig. 6.8.b). Their attainment of G2 phase and active cycling was confirmed by their progress to mitosis in the first 12h of the sampling period and the ensuing nuclear divisions resulted in an increase in cell number between 12h and 24h (Fig. 6.8.a).

6.3.3 Levels of $p34^{cdc2}$-like protein during the cycling of suspension culture cells of *N. plumbaginifolia* previously treated with absence of added hormone, 2,4-D-only, kinetin-only or both 2,4-D and kinetin

Fig 6.3.a shows the change in levels of $p34^{cdc2}$-like protein in 50 µg samples of total cell protein in control cells which, after starvation for both 2,4-D and kinetin for 8 days, were further cultivated in CSV 0 medium for 2 days and then sampled in the subsequent 42h. The content of $p34^{cdc2}$-like protein gradually declined from 12.5×10^5

radioactivity units at 0h to 4 x 10^5 units at 42h. This decline is too steep to have been occurring throughout the 10 day period of hormone starvation before the 42h sampling period. It is probable that the wash of cells with hormone-free medium after 8 days may have further lowered auxin level and initiated the decline. The fact that the content of $p34^{cdc2}$-like protein in the cells is only 12 x 10^5 radioactivity units compared with the 14-15 x 10^5 units present in cells that received some hormone stimulation in the period after the 8 day depletion (Fig. 6.5.a, 6.7.a, 6.9.a) suggests that $p34^{cdc2}$-like protein began to decline when cells were centrifuged and resuspended in hormone free medium after the 8 day depletion. It is likely therefore that this decline was continuing during the sampling period, but it is not clear why the decline should have apparently been so rapid between the 0h and 6h samples (Fig. 6.3.a). No intended change in culture conditions occurred at 0h of the sampling period. Fig 6.5.a shows the levels of $p34^{cdc2}$-like protein in cells that were resuspended in kinetin-only CSV medium for 2 days then supplemented with 2,4-D at the start of the sampling period. The content of $p34^{cdc2}$-like protein per 50 μg of total cell protein remained between 12 x 10^5 to 14 x 10^5 radioactivity units. A similar constancy and absolute level of the protein was observed in cells that were treated with 2,4-D only before sampling (Fig. 6.7.a) and in cells that were provided both 2,4-D and kinetin for 2 days before sampling (Fig. 6.9.a).

These results indicate that in this long established cell line, which has a high endogenous level of $p34^{cdc2}$-like enzyme (Chapter 3) the level of the enzyme remains high and is unresponsive to all but prolonged and stringent depletion of hormone. However, the migration of $p34^{cdc2}$-like protein in cells, which were cultivated in either CSV 0 or kinetin-only CSV medium, in SDS-poly-acrylamide gels, as shown in lane a and b in Fig. 6.10, is faster than that in cells which were cultured in either auxin only CSV medium or complete CSV medium, as shown in lane c and d in Fig. 6.10. These results are consistent with the possibility that the phosphorylation of higher plant $p34^{cdc2}$ follows the pattern seen in yeasts and animal cells, where the protein is not phosphorylated in G0 phase and may not be phosphorylated in G1 phase but during G2 phase becomes phosphorylated on threonine 161/167, on threonine 14 and tyrosine 15 as a prelude to catalytic activition towards mitotic substrate by removal of the latter two

phosphates. According to this pattern cells that are quiescent or arrest in G1 phase, as is the case with cells incubated with no hormone or with kinetin-only, will have unphosphorylated $p34^{cdc2}$. Whereas cells arrested in G2 phase or able to progress through G2 phase, as is the case with cells incubated with 2,4-D-only or with both hormones, will have phosphorylated $p34^{cdc2}$. It has been demonstrated in HeLa cell extracts that phosphorylated $p34^{cdc2}$ migrates more slowly during electrophoresis (Draetta and Beach, 1988) and this has been directly confirmed with ^{32}P labelled $p34^{cdc2}$-like enzyme from the unicellular plant *Chlamydomonas* (John et al., 1989). In the extracts from suspension culture cells studied here the slower-migrating $p34^{cdc2}$-like protein is observed in extracts from cells that have been diagnosed as arrested in, or able to pass through, G2 phase. The implication that $p34^{cdc2}$-like protein may be in different catalytic activity states was therefore examined.

6.3.4 Activity of $p34^{cdc2}$-like protein kinase in non-cycling or synchronised cells after omission or treatment with 2,4-D and kinetin

Control cells that were made quiescent by deprivation of both auxin and cytokinin did not yield significant levels of $p34^{cdc2}$-like H1 histone protein kinase activity. These cells were depleted of hormone for 8 days then spun down and resuspended in hormone-free medium for 2 days, and then sampled in the subsequent 42h (Fig 6.3.b). The lack of obvious activity of $p34^{cdc2}$-like protein kinase in these cells coincides with absence of cell division (Fig. 6.2.a).

Cells that were cultured in kinetin-only medium demonstrated the arrest points at which auxin is required. They were obtained by 8 days of hormone depletion then cells were spun down and resuspended in CSV medium containing 0.23 μM kinetin and were cultured for 2 days, and then supplemented with 9 μM of 2,4-D at time 0h in the subsequent 42h sampling period and sampled in subsequent 42h. During their resumed cell cycle activity two peaks of $p34^{cdc2}$-like protein kinase were detected, one occurring before 12h with a maximum activity of 2 pmoles ^{32}P transferred to histone H1 per gram fresh cells per min and the other with about 1 pmole ^{32}P transferred to histone H1 per gram fresh cells per min at 27h (Fig. 6.5.b). These two peaks of activity correlate with

the two increases in cell number from 0h to 12h and from 24h to 42h (Fig 6.4.a). Consideration of the nuclear DNA profile (Fig. 6.4.b) suggests that the first peak of activity was contributed by the cells in which nuclear DNA content was in G2 phase at time 0h when supplemented with 2,4-D (Fig. 6.4 b), and the second peak was from the cells in which nuclei were in G1 phase at time 0h when supplemented with 2,4-D (Fig. 6.4. b).

However, cells that were cultured in auxin-only indicated a requirement for cytokinin to enter mitosis. These cells were obtained by 8 days of hormone depletion then spinning down cells and resuspending them in CSV medium containing 9 μM of 2,4-D for 2 days, and then supplementing with final 0.23 μM of kinetin at time 0h in the sampling period. Nuclear DNA content indicated that the population at time 0h was predominatly in G2 phase (Fig. 6.6.b). The entire population resumed cycling by activiting $p34^{cdc2}$-like kinase which reached an activity of about 7 pmole ^{32}P transfered to H1 histone per gram fresh weight of cells per minute by 12h (Fig. 6.7.b). This higher peak in activity correlates with homogenous arrest in G2 phase and with progress of all cells in the population through mitosis leading a doubling in cell number by 18h (Fig. 6.6.a). A second cell cycle was then entered and nearly completed during the sampling period, resulting in a second peak of $p34^{cdc2}$-like kinase activity at 27h and a consequent increase in cell number from 30h.

Control cells that were cultured in complete medium after an 8 day period of hormone depletion, were completing their second cell cycle after return to complete medium during the sampling period. Their synchrony had declined but a wide activity peak of $p34^{cdc2}$-like protein kinase from 12h to 24h, with the highest activity of about 6 pmoles ^{32}P transferred to histone H1 per gram fresh cells per min (Fig. 6.9.b), correlated with a concurrent increase in cell number. The early activation of the enzyme and increase in cell number correlate with evidence from DNA content that the cells were already in G2 phase by the beginning of the sampling period (Fig. 6.8.a and b).

Auxin (2,4-D) and cytokinin (kinetin) both contribute to progress through the cell cycle and are therefore directly or indirectly necessary for the activation of $p34^{cdc2}$-like protein kinase at mitosis. Cytokinin may act rather directly on $p34^{cdc2}$-like kinase

activation at mitosis since cells lacking kinetin were arrested in G2 phase with non-activated $p34^{cdc2}$-like kinase and activated it rapidly on stimulation with kinetin (Fig. 6.7.b). It was considered possible that this activation process requires dephosphorylation of $p34^{cdc2}$-like protein kinase because the activity of $p34^{cdc2}$ protein kinase is regulated by phosphorylation and dephosphorylation of $p34^{cdc2}$ (Nurse, 1990).

Direct bichochemical evidence was obtained that supports the hypothesis that $p34^{cdc2}$-like protein, in cells that have been arrested in G2 phase by lack of cytokinin, is in an inactive phosphorylated state that can be activated by removal of phosphate. The acid phosphatase enzyme of potato has been shown to be capable of activating the $p34^{cdc2}$ enzyme of *Xenopus* oocytes (Pondaven et al., 1990). It was tested against the $p34^{cdc2}$-like enzyme of suspension culture cells that had been arrested by cytokinin and a significant *in vitro* activation was observed (Fig. 6.11). The 5 minutes of incubation with phosphatase resulted in a doubling of the catalytic activity of $p34^{cdc2}$-like protein kinase (D-P) that was derived from cells that had been incubated with 2,4-D-only and arrested in late G2 phase (Fig. 6.6.b) due to lack of cytokinin. A much smaller activation was observed with enzyme from cells arrested by cytokinin-only (K-P) that were predominantly not arrested in late G2 phase. The directness with which kinetin may act upon $p34^{cdc2}$-like kinase dephosphorylating enzymes is unknown.

The suspension culture that has been investigated here is extremely useful for identifying cell cycle transitions that are hormone dependent and for investigating the effects of hormones on $p34^{cdc2}$ activation at the G2/M transition. However the cell suspension, which was selected for minimal cell clumping has developed high levels of $p34^{cdc2}$-like protein (Chapter 3) and is presumably less suitable for study of normal changes of accumulation of the protein under hormone influence. The tobacco pith system, in which normal tobacco cells are excised from an intact plant, was therefore also studied.

6.3.5 Resumption of cell division in tobacco Wisconsin 38 Pith tissue induced by NAA and BAP

Tobacco pith is a classic experimental system for the study of hormonal requirements for resumption of cell division. Cell division can be induced by auxin (IAA) and cytokinin (BAP) in differentiated pith parenchyma cells (Jablonski and Skoog, 1954). However, the mechanism by which resumption of cell division is induced is unknown.

As previously observed by Skoog and his collaborators (Jablonski and Skoog, 1954) incubation with the auxin NAA at 1μg/ml, without cytokinin, resulted only in cell enlargement without cell division when blocks of *N. tabacum* cv. Wisconsin Havana 38 pith tissue were incubated on agar MS medium containing 5.4 μM (1mg/l) NAA for 8 days. When the block was incubated for 14 days, nearly all of the cells in it became enlarged (Fig.6.13 bottom right) but none divided. Cell enlargement is reflected in the expansion of the tissue blocks, which can be compared in size with those in the top row of Fig. 6.13, which were on medium lacking auxin and did not expand.

Tissue incubated on agar with both 5.4 μM NAA and 0.56 μM BAP for 8 days showed not only cell enlargement and consequent enlargment of the pith block but also many small foci of callus at the surface. More extensive callus formation, including some that became green in colour, was observed after 14 days (Fig.6.13. bottom left). These calli show both cell enlargement and cell division.

However, neither cell enlargement nor cell division could be seen when the blocks of pith tissue were cultivated on either the hormone free agar MS medium or MS medium containing only 0.56 μM BAP for 14 days (Fig.6.13. top left and right).

These results confirm that the dedifferention of tobacco pith tissue into callus formed by both cell division and enlargment, requires the presence of both cytokinin and auxin; NAA-alone induced only cell enlargement and BAP-alone could induce neither cell enlargement nor cell division in tobacco pith.

6.3.6 DNA synthesis during resumption of cell division in tobacco Wisconsin 38 pith induced by NAA and BAP

In pith that was incubated on hormone-free agar, or agar containing 0.56 μM BAP-only, incubation with BrdU showed failure of incorporation into nuclei, (Fig. 6.14.b, 6.15.b) although the nuclei were detected as irregular bright areas by DAPI stain (Fig. 6.14.a, 6.15.a). The irregular outline of nuclei in these large differentiated cells contrasts with the more spherical nuclei in the smaller proliferating cells (Fig. 6.17.a). Positive detection of BrdU incorporation results in brightly fluorescing nuclei as can be seen in Fig. 6.17.b. In pith tissue cultured on agar containing only 5.4 μM of NAA for 20 days no DNA synthesis could be detected by incorporation of BrdU into the nuclei, although these cells became enlarged (Fig. 6.16.b); and the nuclei could be stained with DAPI (Fig. 6.16.a). Test for BrdU incorporation made at 8 and 14 days of incubation with NAA-only also showed zero incorporation (not shown). It is expected that some DNA synthesis may have occurred in freshly isolated pith due to wound response (Simard, 1971; Patau et al., 1957) but labelling at early times was avoided here. By frequent sampling in the first few days after excision Simard (1971) considered that he identified an initial period of DNA synthesis due to wound response followed, after 3 days, by a period in which DNA synthesis could be accelerated by the provision of auxin alone, presumbly while the wound response was subsiding. The present study allowed the wound response to subside fully and the, presumeably transiently-elevated, levels of several hormones that are part of that response to also decline, before testing the effect of individual and mixed hormone supplementations. Under these conditions no stimulation of DNA synthesis could be induced by single hormones (Fig. 6.15, 6.16). It is probable that by 8 days of incubation on auxin-only (Fig. 6.16) cells were arrested in a cell cycle phase (or phases) from which they could not be induced to resume cycling by individual hormones. The behaviour of suspension culture cells (Chapter 6.3.4) indicates that this is possible since cells in G2 phase require both auxin and cytokinin to proceed. However, in the callus which was cultivated on the agar MS medium containing both 5.4 μM of NAA and 0.56 μM of BAP DNA synthesis could be detected by incorporation of a BrdU pulse into about 80% of nuclei at day 8 (Fig. 6.17.b) and about 30% of cells still

could be detected as still cycling and carrying out DNA synthesis at day 20 (Fig.6.17. a and b; Fig. 6.18).

One interesting observation is that only large nuclei were present in the pith tissues that were cultured on hormone free MS medium, or MS medium containing either NAA or BAP alone (Figs. 6.14.a, 15.a, and 16.a), and many small nuclei could be detected in pith callus, which was cultured on MS medium containing both NAA and BAP (Fig. 6.17.a). The larger nuclei correlated with the larger size and non-dividing nature of the cells in medium lacking both NAA and BAP.

These results indicated that the DNA synthesis only occurs in the pith callus which is cultivated on the MS medium containing both NAA and BAP and confirms that dedifferentiation of tobacco pith tissue requires the combined stimulation of both auxin and cytokinin.

6.3.7. Levels of $p34^{cdc2}$-like protein in excised pith induced by NAA and BAP

Levels of $p34^{cdc2}$-like protein relative to total protein were found to be low in freshly excised pith parenchyma cells whether from upper (I_u) or lower (I_l) regions of the stem (Fig. 6.19.I_u and I_l) relative to the levels that developed when the cells were stimulated to resume division by incubation on solid medium with added phytohormones (Fig. 6.19. M, B, N and N + B).

Tissue that was excised and incubated without added hormone showed a small increase in level of $p34^{cdc2}$-like protein (Fig. 6.19.M) relative to initial levels (I_u, I_l), which correlated with the occurrence of wound response-associated cell cycle activity in the first 3 days after excision (Simard 1971). To allow activity that derived from wound response to subside fully sampling was delayed until 8 days after excision. To investigate the possibility that recent division activity might influence the pith respondence, tissue was excised from close to the stem apex since this has divided more recently than pith close to the stem base. Pith responded homogenously in terms of accumulating $p34^{cdc2}$-like protein, regardless of its origin within the stem and therefore regardless of how recently it had previously divided. On medium without added hormones, pith from upper

stem (Fig. 6.20.a, treatment 1, 2, 3) or lower stem (treatment 4, 5, 6) retained the low levels of $p34^{cdc2}$-like protein that were characteristic of pith tissue that has been isolated in non-dividing state and has slightly increased its $p34^{cdc2}$-like protein levels (Fig. 6.19) presumably due to the transient wound response that was presumbly strongest in the surface layer. Incubation of pith from the same regions in medium supplemented only with 0.56 μM BAP produced no greater increase in $p34^{cdc2}$-like protein level (Fig. 6.20. a and b, treatments 7, 8 and 9), which remained at close to 20,000 radioactivity units (note the different scaler in Fig. 6.20.a and c). However pith from the same regions when incubated in parallel but on medium containing 5.4 μM NAA (Fig. 6.20. c and d treatment 7, 8 and 9) $p34^{cdc2}$-like protein was induced more than tenfold to levels close to 250,000 radioactivity units. The combined presence of both auxin and cytokinin hormones did not result in any greater induction than did auxin alone. The presence of sustained cell proliferation activity was dependent upon the presence of both types of hormone but the electrophoretic pattern did not show any features specific for this condition. Presence of NAA, which induced $p34^{cdc2}$-like protein levels more than tenfold, correlated with the presence of slower-migrating PSTAIR protein. One possibility is that this correlates with a more extensively phosphorylated form of the protein. Such a form has been observed in the G2 phase of animal cells (Draetta and Beach, 1988) and a similar form was detected in *Chlamydomonas* (John et al., 1989).

6.3.8 Activity of $p34^{cdc2}$-like protein kinase in excised pith treated with NAA and BAP

6.3.8.1 Recovery of $p34^{cdc2}$-like protein kinase from pith tissue

Activity of $p34^{cdc2}$-like protein kinase was present in tobacco pith that had been induced by NAA together with BAP and its presence correlated with cell proliferation that was detected by BrdU. The $p34^{cdc2}$-like protein kinase was affinity purified with $p13^{suc1}$-Sepharose 4B beads, eluted with free $p13^{suc1}$ from the beads and its catalytic activity was measured using histone H1 as substrate. Fig. 6.22 shows that the recovered activity of $p34^{cdc2}$-like protein kinase increased as the fresh weight of pith callus taken for extraction increased. However, the recovered activity of $p34^{cdc2}$-like protein kinase

increased only slightly when the fresh weight of pith callus exceeded 0.3g in these experiments, in which 30 µl of $p13^{suc1}$ beads, containing 8 mg $p13^{suc1}$ per ml of beads, were used to couple $p34^{cdc2}$-like protein kinase, 50 µl of 0.5 mg/ml of free $p13^{suc1}$ were used to elute $p34^{cdc2}$-like protein kinase from the beads. Amounts of tissue less than 0.3g were therefore used in subsequent experiments to measure the activity of $p34^{cdc2}$-like protein kinase in pith.

6.3.8.2 The activity of $p34^{cdc2}$-like protein kinase in pith treated with NAA and BAP.

The combined presence of 5.4 µM NAA with 0.56 µM BAP, which resulted in sustained cell proliferation (Fig. 6.17), also resulted in the presence of the catalytically active form of $p34^{cdc2}$-like protein, as shown in Fig. 6.23. Although NAA when present alone was able to induce $p34^{cdc2}$-like protein to levels as high as those that resulted from the combined presence of NAA and BAP (Fig. 6.20.c and d) the protein was not catalytically active as a protein kinase with the mitotic substrate H1 histone. Slightly higher activity levels were detected for all hormone treatments from pith tissue taken from the upper stem. It is possible that upper stem pith is more sensitive to the inducement of hormone than lower stem pith because upper stem pith is younger and more recently engaged in cell division. However a contributory factor may be that older pith contains a greater proportion of cell wall and a correspondingly lower proportion of protein, therefore measurements made on a fresh weight basis will represent smaller amounts of total protein from older pith.

6.3.8.3 Individual variation between plants of inducibility of $p34^{cdc2}$-like protein kinase in excised pith

Individual plants of tobacco Wisconsin 38 were highly reproducible in the response of the excised pith to MS agar medium containing both NAA and BAP (Fig.6.24), and consistently unresponsive in development of $p34^{cdc2}$-like protein kinase when cultivated on either the hormone free MS medium, medium containing NAA only, or medium containing BAP only.

6.3.8.4 Time course of change in level of p34^{cdc2}-like protein kinase activity during the incubation of tobacco pith with mixtures of NAA and BAP

Fig 6.25 shows the activity of p34^{cdc2}-like protein kinase in the pith callus, which was from the same plant and incubated on the same culture medium, when sampled at different times during incubation. The activity of p34^{cdc2}-like protein kinase in the pith callus cultivated on the MS medium containing both NAA and BAP was the highest at day 8 (about 2.8 pmoles ^{32}P transferred to histone H1 per gram fresh weight pith callus per min), and then gradually declined at day 14 (about 2.5 pmoles ^{32}P transferred to histone H1 per gram fresh weight pith callus per min) and at day 20 (1.8 pmoles ^{32}P transferred to histone H1 per gram fresh weight pith callus per min). The decline of activity of p34^{cdc2}-like protein kinase implies that the number of actively cycling cells decreased, but it does not mean that these cells had lost the ability to divide. It is possible that the callus had begun to exhaust nutrient or hormones through the period between 8 to 20 days. Only basal levels of p34^{cdc2}-like protein kinase were detected in pith tissue that was incubated on MS 0, medium containing only BAP, or medium containing only NAA over the whole period 8 to 20 days.

6.4 Discussion

6.4.1 Requirement of auxin and cytokinin at specific cell cycle transitions by *N. plumbaginifolia* cells in suspension culture

Van't Hof noted that cells in plant meristems cells arrest in G1 and G2 phases when they cease division (Van't Hof 1974) and he therefore hypothesized that principle control points were located in these phases. Parallels with other eukaryotes in which the role of the *cdc* gene product has been better characterised has lead to the suggestion (John et al., 1989, 1993b) that Van't Hof's principal control point hypothesis should be refined to recognise late G1 START and mitotic initiation as the control points in G1 and G2 phases. In the late G1 period following the START division event biochemical processes leading to DNA replication occur. In this period there must be either synthesis or activation of the components necessary for DNA replication, for example, endonucleases

that introduce single-strand nicks into double-stranded DNA, DNA polymerases, DNA ligases, DNA-dependent ATPase and DNA binding proteins. The earliest of these events may well be the activation of $p34^{cdc2}$ protein kinase, although its substrates at START are unknown. (Chapter 3; Nurse and Bissett, 1981; Beach et al., 1982; Krek and Nigg, 1991a).

As in animal cells the relationship between cell growth and cell division remains one of the least understood areas (Norbury and Nurse 1992). In animal cells it is unclear whether most oncogenes directly stimulate cell growth and only secondarily result in division by leading to attainment of the necessary cell size. Similarly in plant cells, hormones that may influence cell division also stimulate growth and in recognition of this they are sometimes termed "growth substances". Classical studies of the requirements of plant cells for proliferation *in vitro* have indicated that at least the auxin class of phytohormones must be present (reviewed by Bayliss, 1985). Although some cell types can be cultured without further hormone supplements the requirements of many cells for cytokinin in addition to auxin (reviewed by Skoog and Miller, 1957) suggests that cells that are independent of externally supplied cytokinin may be synthesising this hormone endogenously. Early attempts to analyse the roles of these two classes of hormone were made in Skoog's laboratory using the excised stem pith parenchyma of tobacco plants. In early experiments with this tissue there was no clear evidence for specific influence of the two classes of hormone on particular cell cycle events. This absence of evidence for dependence of specific events upon particular hormones may have derived from analysis of freshly excised tissue, in which wound response leads to cell cycle activity. In such freshly excised tissue both auxin and cytokinin were found to stimulate the rate of both DNA synthesis and of mitosis (Patau et al., 1957) but this apparent non-specifity of hormone action may have derived from raised endogenous levels of several hormones during the wound response. An unusual early observation from Skoog's laboratory was that a siginificant number of cells in tissue stimulated with auxin alone became multinucleate and this therefore seemed to implicate the missing cytokinin as an agent necessary for post mitotic events at cytokinesis (Naylor et al., 1954). The name of this class of hormones reflects this apparent cytokinesis function, although the phenomenon

of multinucleate cells being induced by lack of cytokinin has rarely, if ever, been reported subsequently. A clear indication of the respective roles of auxins and cytokinin in tobacco stem cells has come from analyses of Simard (1971), who allowed the wound response to subside for 3 days before investigate the effects of individual hormones. He then observed that auxin alone, but not cytokinin alone, could stimulate DNA synthesis. Nonetheless evidence for specific effects of hormones particularly at mitosis has remained inconclusive (reviewed by Bayliss, 1985).

The present observations made with hormone-limited suspension culture cells are therefore siginificant because they indicate that, while auxin is necessary for both progress from G1 to S phase and also from G2 to M, cytokinin is stringently required specifically at the G2/M transition. It is also sigificant that for the first time the point at which cells arrest when cytokinin level is inadequent is seen to be just prior to the activation of $p34^{cdc2}$-like kinase in late G2 phase. Cells arrested for lack of cytokinin are clearly in G2 phase from evidence of their nuclear DNA content (Fig. 6.6.b) and, on provision of cytokinin, they rapidly activate $p34^{cdc2}$-like H1 protein kinase (Fig. 6.7.b) and pass through nuclear division to formation of daughter cells (Fig. 6.6.a). The arrest point of tobacco pith cells when provided with auxin but not cytokinin may well be similar since such cells are indicated by the results of Simard (1971) to have replicated DNA but they are unable to continue to do so (Fig. 6.16) and are therefore blocked in the cell cycle, presumably in G2 phase.

The data presented in this thesis are consistent with those of Meyer and Cooke (1979) who studied division in tobacco mesophyll cells recovering after protoplasting. In these cells DNA synthesis could be induced by auxin (2,4-D) alone but an eventual addition of cytokinin (6BA) was required for completion of the cell cycle. Interpretation of this effect of cytokinin is complicated by a requirement for transfer to fresh medium at the time of cytokinin addition, which indicates a possible contribution from removal of unknown inhibitors. A closer parallel with data reported here is provided by the observations of Jouanneau et al (1973) on suspension culture cells of *N. tabacum* c.v. Wisconsin 38 (identical with the cultivar that was used for pith studies in this thesis but different from the suspension culture). In *N. tabacum* culture cells that were

resuspended (but not washed) in medium lacking added cytokinins DNA synthesis could be stimulated by 2,4-D without added cytokinin and a third of the cells continued to mitosis, perhaps due to carried-over cytokinin. Cells that were 2,4-D stimulated but remained undivided were presumably in G2 phase but this was not determined. Evidence was obtained that cytokinins might induce preparations for mitosis long before the event since cells that were arrested by resuspension in medium without cytokinins, when treated for 19h with cytokinin (IPA) could then continue through mitosis even if resuspended in cytokinin-free medium. However the hypothesis (Jouanneau et al., 1973) that this indicates the induction by cytokinins of all necessary preparations for mitosis long before the event is not convincing since the cells were transferred to cytokinin-free medium without washing, therefore carry-over of cytokinin may have been sufficient to explain the continuing mitotic stimulation. Interestingly, the minority of cells that succeeded in mitosis after transfer to nominally cytokinin-free medium progressed four times slower than normal through metaphase. Maximal activity of $p34^{cdc2}$ is normally present at metaphase (Moreno et al., 1989) and the slow progress of metaphase in suboptimal cytokinin may indicate suboptimal activation of $p34^{cdc2}$-like enzyme since the data in this thesis indicates that presence of cytokinin is required for its normal activation.

Both suspension culture cells and pith explant cells, that have auxin provided but lack cytokinin, have low activities of $p34^{cdc2}$-like kinase (Figs. 6.7.b, 6.22, 6.23) although high levels of $p34^{cdc2}$-like protein are present (Figs. 6.7.a, 6.20.c and d). In the suspension culture cells nuclear DNA content directly indicated that they were in G2 phase. In this cycle phase the $p34^{cdc2}$ protein of yeast and animal cells becomes phosphorylated at threonine 161 (or the equivalent threonine, which is at 167 in mammalian $p34^{cdc2}$) together with phosphorylation at tyrosine 15 and also in mammalian $p34^{cdc2}$ at threonine 14. In this multiply-phosphorylated form the enzyme is bound to cyclin B and is catalytically inactive, but it can be activated at the initiation of mitosis by dephosphorylation of tyrosine 15, and also dephosphorylation of threonine 14 if as in higher eukaryotes it has been phosphorylated at that position, leaving the active form that is phosphorylated at only threonine 161/167 (reviewed by Norbury and Nurse 1992).

Phosphorylation of $p34^{cdc2}$ is a characteristic of cycling cells since quiescent cells that remain in G0 phase have unphosphorylated $p34^{cdc2}$ (Simanis and Nurse, 1986) whereas phosphorylation at threonine 161/167 is characteristic of $p34^{cdc2}$ catalyticaly active in G2 phase.

The experimental observations reported here are consistent with a similar type of control of $p34^{cdc2}$ activity in higher plant cells. An increase in phosphorylation state of $p34^{cdc2}$-like protein is indicated by a reduced electrophoretic mobility, which has been observed in both human cells and in the unicellular plant *Chlamydomonas* (Draetta and Beach, 1988; John et al., 1989) and has been correlated with increased presence of radioactive phosphrous on $p34^{cdc2}$ protein. The present study did not attempt ^{32}P labelling of $p34^{cdc2}$ but a consistent correlation of reduced electrophoretic mobility of $p34^{cdc2}$like protein was observed in suspension culture cells when either arrested in G2 phase in the presence of auxin without cytokinin or when able to pass through G2 phase in the presence of both auxin and cytokinin (Fig. 6.10.c and d). Interestingly, in pith tissue as well, a shift to lower mobility forms of $p34^{cdc2}$-like protein (Fig. 6.20. b and d) correlated with presence of auxin-only and therefore with probable arrest in G2 phase, and the shift also correlated with active proliferation in the presence of both auxin and cytokinin (Fig. 6.17) and therefore with cells passing through G2 phase. In yeast and animal cells the $p34^{cdc2}$ protein is phosphorylated in G2 phase, as explained above. The data from plant cells must be interpreted with caution however for four reasons. Firstly phosphorylation of $p34^{cdc2}$ was not demonstrated directly. Secondly little difference in mobility profile was observed between enzyme taken from cells arrested in G2 phase and cells that were actively cycling. It might have been expected that the presence of some cells in G1 phase in the latter sample could have resulted in some difference of pattern. One possibility is that, as in chick (Krek and Nigg, 1991a), the plant $p34^{cdc2}$ enzyme is phosphorylated during G1 phase. The chick enzyme is reported to be phosphorylated on serine 277 during G1 phase. In that case the clearest difference would be between non-cycling cells in G0 phase, which have non-phosphorylated $p34^{cdc2}$ (Simanis and Nurse, 1986), and cells that have entered the division sequence G1-S-G2-M and are either arrested or progressing. Therefore enzyme from cells arrested in the presence of auxin-

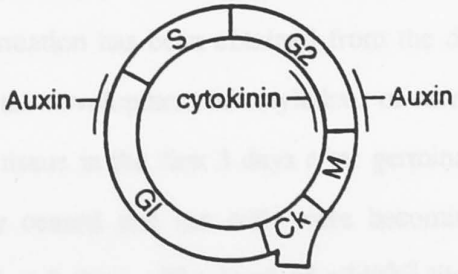
only or actively cycling would in each case be predominantly phosphorylated. Thirdly, in the synchronous cultures established by hormone deprivation, changes in band pattern of $p34^{cdc2}$-like protein were not clearly observed to correlate with phases of the cell cycle (Fig. 6.5.a and Fig. 6.7.a). It is again possible that the enzyme is phosphorylated in all phases of actively cycling cells although at different sites, and therefore its mobility changes rather little, except between G0 and cycling cells. Cells lacking auxin may well be in G0 phase. Finally the pattern of mobility in $p34^{cdc2}$-like protein is different when taken from suspension culture or from pith cells. From suspension culture cells the enzyme is predominantly of higher mobility from cells that have been cultured without auxin and is predominantly of lower mobilty from cells with auxin (Fig. 6.10). Whereas enzyme from pith without auxin shows significant amounts of both slower and fast migrating enzyme (Figs. 6.20, 6.21) and predominantly only the slower-migrating form in auxin treated tissue. The difference between suspension cell and pith may lie in the state of the cells at the beginning of the experiments since suspension cultured cells were brought to stationary phase by hormone depletion and they arrested largly in G1 phase, whereas cells that were excised from the intact plant were in the cell cycle phase that was determined by the developmental program of pith formation within the plant. An unknown proportion of the pith cells when excised may have been in G0 phase and they may have abstained from entering G1 in the absence of auxin. Therefore the two mobility forms of the $p34^{cdc2}$like protein in cells not treated with auxin might be accounted for.

A less indirect indication that cells that have been limited by absence of cytokinin, which have arrested in G2 phase, do contain phosphorylated $p34^{cdc2}$ that can be activated to catalytic function by dephosphorylatioon, has come from the ability of phosphatase enzyme to activate isolated $p34^{cdc2}$-like enzyme *in vitro* (Fig. 6.11). A activation was observed and a greater activation may be difficult to obtain by use of a non-specific phosphatase. The enzyme that is normally responsible for activating $p34^{cdc2}$ is cdc25, which is a specialised phosphoprotein phosphatase that recognises $p34^{cdc2}$ and removes phosphate from both Thr 14 and Tyr 15. The phosphate on Thr 161/167 is not removed by cdc25 and is necessary for activity of $p34^{cdc2}$ (Millar et al., 1991). The

$p80^{cdc25}$ enzyme is not available as an experimental reagent and the acid phosphatase that was used instead is non-specific in action. It is expected to remove phosphate groups at random and any molecule of $p34^{cdc2}$ that loses phosphate from Thr 161/167 will be inactivated whereas activation will require chance removal of both Thr 14 Tyr 15 phosphate. Therefore the presence of the activated form of $p34^{cdc2}$ will be partial and transient.

The available evidence therefore suggests that cytokinin together with auxin is necessary for activation of $p34^{cdc2}$-like protein kinase at plant mitosis. As with all hormone mediated effects in plants the molecular events immediately following hormone reception are unknown, but in the case of cytokinin they lead rapidly to mitotic activation of $p34^{cdc2}$-like kinase and, by comparison with other eukaryotes and from the evidence of *in vitro* activation by phosphatase, this may be catalysed by a cdc25-like phosphatase. Auxin makes more extensive contributions to the division cycle, being necessary both for progress from G1 phase and also jointly with cytokinin for progress from G2 phase. In the later role it may participate with cytokinin in the pathway leading to cdc25 dephosphorylation of $p34^{cdc2}$.

The regions of most stringent hormone control of the cell cycle in *N. plumbaginifolia* suspension cells can be summarised by the following diagram.



Data from tobacco pith tissue are fully consistent with the operation of similar controls. It should be noted however that both of these cell types derive from the aerial parts of the plant and that, although low concentrations of cytokinin are conducive to root cell division (reviewed by Skoog and Miller, 1957; Hamill 1993) and therefore the diagram may be wide application, it does not deal with the phenomenon of inhibition by supra-optimal concentrations of hormone, as can occur in root tissue at concentrations of

cytokinin that may not be inhibitory in stem tissue (Torrey, 1962; Wightman and Thimann, 1980; Wightman et al., 1980).

6.4.2 $p34^{cdc2}$-like protein in the switch between division and differentiation in higher plants

Data from tobacco suspension cultures and pith tissue also have a bearing on the possible mechanism by which cells exit from cell division activity and are able to enlarge and differentiate.

In higher plants, new cells that continually arise in meristems by cell division usually stop dividing and develop to specialised organs and tissues. For example leaves of wheat seedlings have a linear developmental gradient from a meristematic region at the base through a series of progressively differentiating leaf blade cells to a mature zone extending upwards to the tip (Boffey et al., 1979). Levels of $p34^{cdc2}$-like protein are consistent with its participation in the control of this developmental program. High levels of $p34^{cdc2}$-like protein at the leaf base correspond with a high percentage of mitotic cells, the levels of $p34^{cdc2}$-like protein declines as cell differentiation proceeds, and the basal level of $p34^{cdc2}$-like protein in fully differentiated cells, which lose the capacity to divide, is one-sixteenth of that in dividing cells (John et al., 1990). Further evidence consistent with the proposition that decline in $p34^{cdc2}$ level is part of the switch from division to differentiation has been obtained from the dicotyledonous plant carrot (*Daucus carota* L.). In the development of cotyledons of carrot, cell division was evenly distributed through the tissue in the first 3 days after germination. From day 4 the cell division had completely ceased and the cells were becoming much larger and more vacuolate than in the first 3 days. The level of $p34^{cdc2}$-like protein relative to other protein was highest at day 1 after germination and then rapidly fell off as the cotyledons expanded, declining by the fourth day to 6% of the level present at the earliest sampling time in differentiated cells. $p34^{cdc2}$-like protein never completely disappeared and remained at the low levels attained at day 4 (Gorst et al., 1991).

To test the possibility that a decline to low levels of $p34^{cdc2}$-like protein is an inevitable secondary consequence of the cessation of division, suspension culture cells of

carrot were arrested in division by nitrogen deprivation with continued supply of 2,4-D. Although many proteins were degraded, and protein per fresh weight declined to one quarter, $p34^{cdc2}$-like protein was retained and this was consistent with the possibility that the continued developmental stimulus to divide that was provided by the auxin analogue caused a retention of the key division protein. This retention revealed that $p34^{cdc2}$-like protein is not inherently unstable in non-dividing cells (Gorst et al., 1991). This conclusion is supported by the present evidence from tobacco suspension culture cells, which retained $p34^{cdc2}$-like protein until thoroughly depleted of auxin, although they ceased division soon after the first decline auxin level (Figs. 6.1.curve 3, 6.7.a) Therefore where a low level of $p34^{cdc2}$ develops it can be seen as a positive developmental phenomenon (reviewed by John and Wu, 1992; John et al., 1993a) rather than a simple consequence of cesssation of division.

Further correlative evidence has come from pea root, where $p34^{cdc2}$-like protein levels are high in the apical meristem and low in the region of cell differentiation behind it (John et al., 1993a). The siginificance of decline in $p34^{cdc2}$-like protein level for the cessation of division is indicated by the consistent requirement, prior to the resumption of division in differentiated plant tissues, for the restoration of $p34^{cdc2}$-like protein levels related to other proteins up to the level seen in meristem cells. This phenomenon was first reported in cotyledon tissue (Gorst et al., 1991) but has also been noted in root tissue (John et al., 1993a) and is now extended to a further different tissue; the stem pith of tobacco.

The results in this chapter showed that tobacco pith which is free of endogenous phytohormones could be induced by both NAA and BAP to dedifferentiate and form callus (Fig. 6.12. bottom left), which could synthesise DNA and divide indicated by the incorporation of BrdU into DNA of nuclei seen with DAPI (Fig. 6.17). Auxin (NAA) when present alone could induce tobacco pith cells to enlarge (Fig. 6.12. bottom right) but no DNA synthesis could be detected (Fig. 6.16). The content of $p34^{cdc2}$-like protein induced either by both NAA and BAP or by NAA-alone was tenfold higher than that in the pith tissue incubated on either hormone-free MS medium or MS medium containing only BAP (Fig. 6.18). The data from tobacco pith are significant in showing that, while

auxin may be sufficient to induce p34^{cdc2}-like protein accumulation, the combined presence of cytokinin may be necessary to allow it to support cell division. The content of p34^{cdc2}-like protein in tobacco pith tissue which was induced by NAA is more than 10 times higher than that in tissue treated with BAP alone (Fig. 6.18) and correlates with the content of p34^{cdc2}-like protein in carrot cotyledon callus induced by 2,4-D which is 16 times higher than that in non-dividing carrot cotyledon cells (Gorst et al., 1991). A similar contribution from cytokinin was probably made in the cotyledon and root tissue investigated earier, as discussed, but was not apparent because of an adequate level of endogenous cytokinin. The capacity to investigate the specific requirement for cytokinin, in inducing the resumption of division in dedifferentiating higher plant tissue, allowed a test of the molecular basis of the phenomenon by which p34^{cdc2}-like protein might be induced in the absence of cytokinin without division occuring. In the presence of auxin without cytokinin the p34^{cdc2}-like protein kinase did not become catalytically active. No appreciable activity of p34^{cdc2}-like protein kinase could be detected in pith tissue which was cultured on MS medium containing only NAA (Figs. 6.23, 6.24 and 6.25). High activity of p34^{cdc2}-like protein kinase could only be detected in pith callus which was induced by both BAP and NAA (Figs. 6.23, 6.24, 6.25) although the levels and migrating pattern in SDS gel of p34^{cdc2}-like protein in pith tissue induced by NAA alone were the same as in pith callus induced by both NAA and BAP. These results showed that the presence of auxin is required for synthesis, and possibly the phosphorylation, of p34^{cdc2}-like protein kinase and that the presence of cytokinin in combination with auxin is required for the activation of p34^{cdc2}-like protein kinase.

The evidence reviewed here is fully consistent with the proposition (John et al., 1990; Gorst et al., 1991; John and Wu, 1992; John et al., 1993a and b) that synthesis and activation of p34^{cdc2}-like protein kinase is an essential component of the resumption of cell division in differentiated plant cells.

Fig. 6.1.

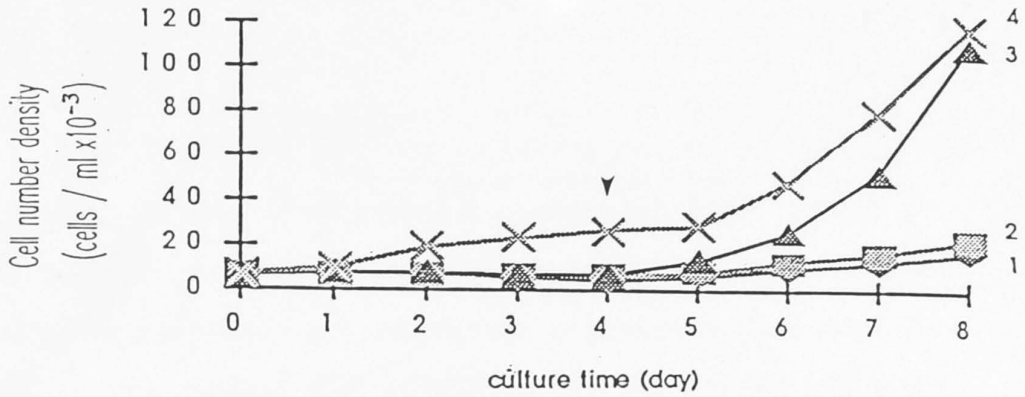
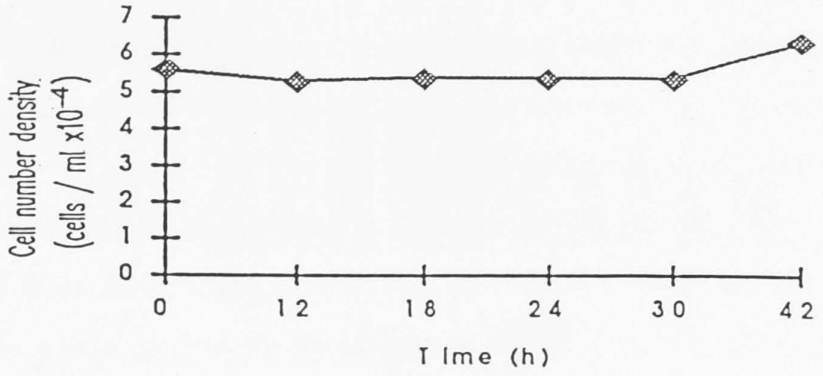


Fig. 6.1. Effects of prior phytohormone depletion and resupply during the sampled period on cell number density of cell suspension culture of *N. plumbaginifolia*. Levels of both auxin and cytokinin were depleted in two consecutive 4 day periods prior to sampling. During the sampling period, in the first 4 days cells were resuspended in; 1 (▽), CSV medium with no hormone (CSV 0); 2 (■), cytokinin (kinetin) only; 3 (▲), auxin 2,4-D only; 4 (×) auxin and cytokinin both present. After 4 days in the sampling period (indicated by vertical arrow) all cultures were supplemented to contain both auxin and cytokinin by addition of both to treatment 1, auxin to 2 and kinetin to 3. When present 2,4-D was supplied at 9 μM and kinetin at 0.23 μM. Cells had previously been cultured for 8 days, without added phytohormone, during a hormone-depletion regime. Cells entering the 8 day depletion period had previously been grown in complete CSV medium containing 9 μM 2,4-D and 0.23 μM kinetin. In this complete medium they were diluted at 4 day intervals from final cell densities of about 6 x 10^6 per ml to one sixth of that density. Cells entering the 8 day hormone depletion period were washed twice by centrifugation in medium without added hormone then resuspended in this medium to the standard initial culture density of 1 x 10^6 cells per ml. After 4 days they were again given the standard dilution to one sixth density in the same hormone-free medium but without centrifugation therefore some carrying over of hormone that may have been leached into the medium during the first 4 days was possible. After the depletion treatment of 8 days without added hormone, the cells were spun down and resuspended in (1) hormone-free CSV 0; (2) 2,4-D-only CSV medium; (3) kinetin-only CSV medium and (4) complete CSV medium respectively, at about 8 x 10^3 cells per ml.

Fig. 6.2. Effect of resuspension in medium lacking auxin and cytokinin (CSV 0 medium) on (a) cell number density and (b) nuclear DNA content. The culture described in this Figure acted as a control for other cultures (Figs.6.4-6.9) that were treated with hormone mixtures for 2 days after the 8-day hormone depletion regime. The culture described here was centrifuged after the 8 day hormone depletion as described in Fig. 6.1. and resuspended in fresh hormone-free medium. At this point an additional decline in hormone concentration probably occurred since cells entering the 8 day depletion regime will have carried over hormone as internal pools that may later have leached or equilibrated with the medium. Discarding the medium by centrifugation at the end of the 8 day period will have discarded any initially carried-over hormone that may subsequently have been leached from the cells. Two days after resuspension (10 days after beginning the hormone depletion regime) the cells were sampled to determine nuclear DNA content, seen in (b). This sampling is equivalent in timing to that in Fig. 6.4-6.9. Sampling was then continued for 42h to determine cell number density, seen in (a) and also levels and activity of $p34^{cdc2}$-like protein kinase seen in Fig. 6.3. During the 2 day presampling period other test cultures (Fig. 6.4-6.9) were incubated with single hormones or a mixture and then during the 42h sampling period were supplemented to provide the complete hormone mixture.

Fig. 6.2.

(a)


Cell number density
(cells / ml x10⁻⁴)
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6
5
4
3
2
1
0
0
12
18
24
30
42
Time (h)

(b)

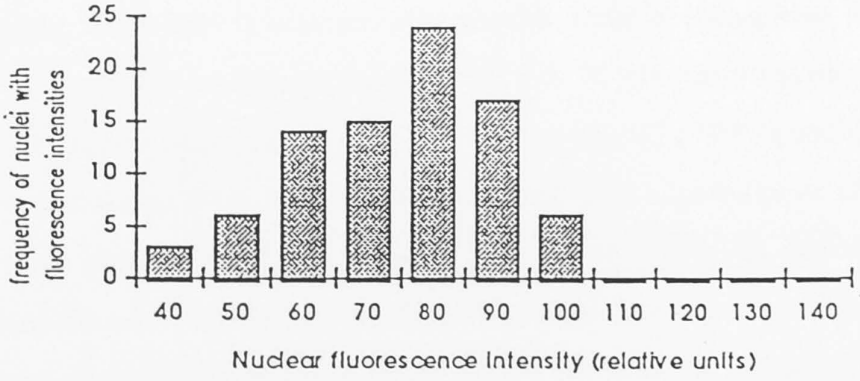
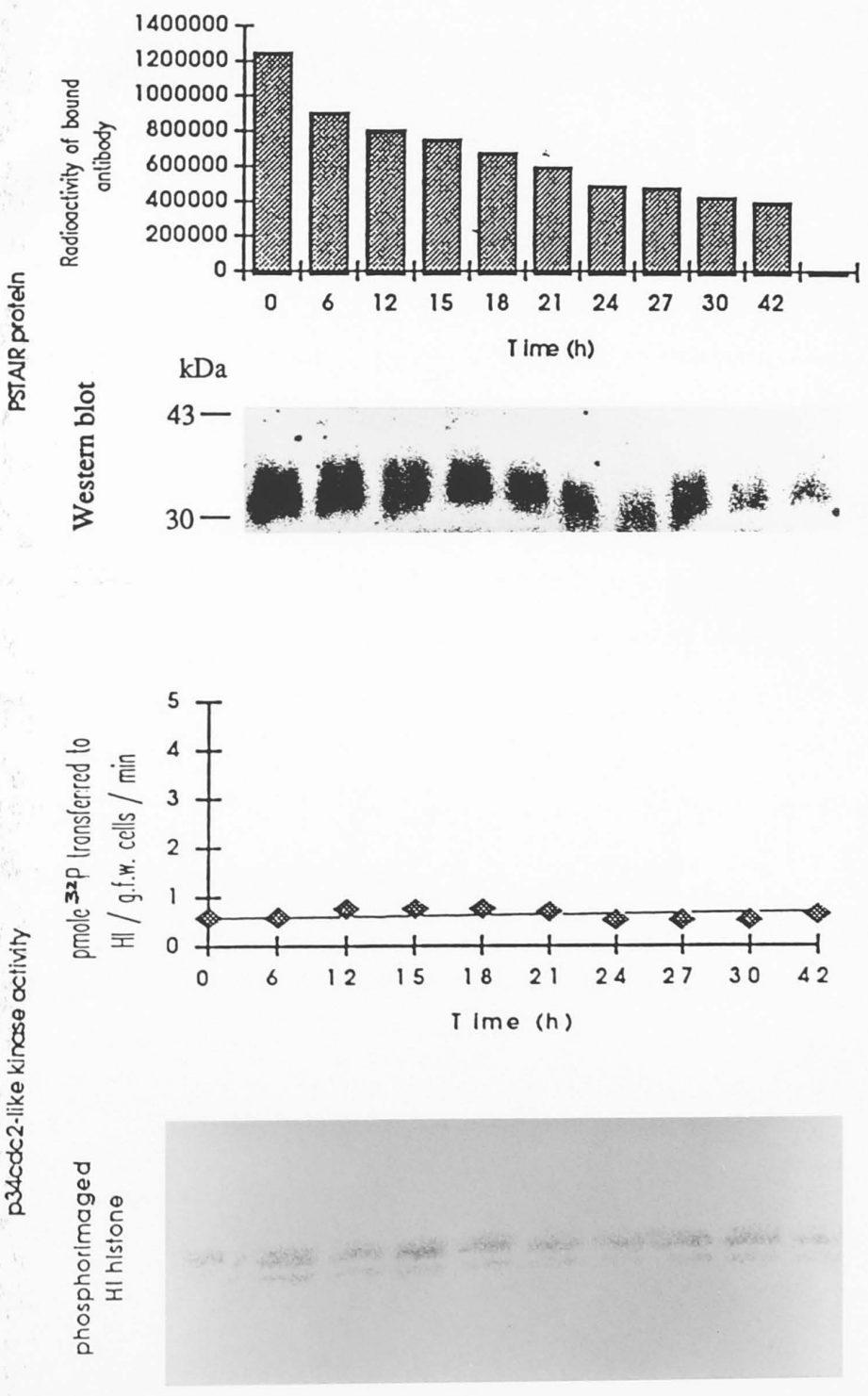

frequency of nuclei with
fluorescence intensities
25
20
15
10
5
0
40
50
60
70
80
90
100
110
120
130
140
Nuclear fluorescence intensity (relative units)

Fig. 6.3. Cells shown in Fig. 6.2, which were in medium lacking auxin and cytokinin, were also sampled for (a) content of $p34^{cdc2}$-like protein and (b) affinity purified $p34^{cdc2}$-like H1 kinase activity.

For estimation of $p34^{cdc2}$-like protein (PSTAIR protein) in the cell suspension culture, equal loadings of 50 µg protein were separated on 10-15 % linear gradient acrylamide gel. For Western blotting transferred proteins were probed with affinity-purified polyclonal anti-EGVPSTAIREISLLKE antibody and the bound antibody was detected by ^{125}I anti-rabbit IgG. The image shown was obtained by exposure in a phosphorImager and also was analysed to determine levels of $p34^{cdc2}$-like protein by quantification of bound isotope shown graphically.

For measurement of the activity of $p34^{cdc2}$-like protein kinase, 0.1g fresh weight of cells ground in liquid nitrogen were extracted in NDE buffer (described in general methods), purified by 20 µl of $p13^{suc1}$ beads, washed and eluted with 50 µl of 0.5 mg/ml of free $p13^{suc1}$. The activity of $p34^{cdc2}$-like protein kinase was measured using Histone H1 as substrate at 30°C for 5 min. The amount of ^{32}P transferred was measured by placing 20 µl of reaction mixture on P81 phosphocellulose paper washing with phosphoric acid then counting in a scintillation counter. The radioactivity of labelled Histone H1, shown at the bottom of the figure, was obtained by separation 30 µl of reaction mixture on a 12% acrylamide gel and then exposure in a phosphorImager.

Fig. 6.3.


(a)
PSTAIR protein
Radioactivity of bound antibody
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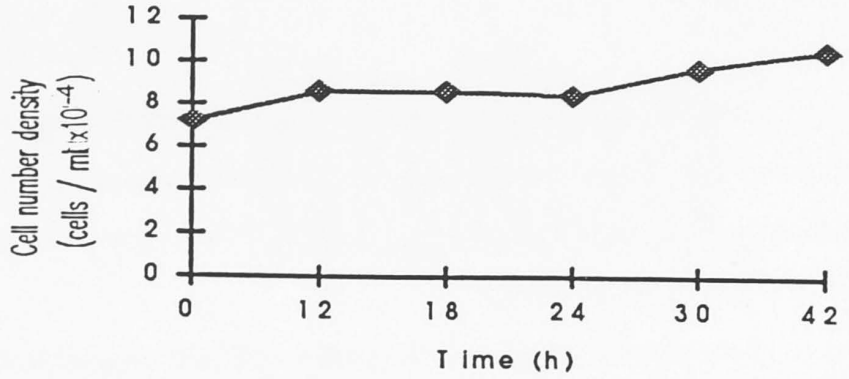
1200000
1000000
800000
600000
400000
200000
0
0 6 12 15 18 21 24 27 30 42
Time (h)
Western blot
kDa
43
30
(b)
p34cdc2-like kinase activity
pmole 32P transferred to HI / g.f.w. cells / min
5
4
3
2
1
0
0 6 12 15 18 21 24 27 30 42
Time (h)
phosphorimaged HI histone

Fig. 6.4. Effect of 2 days treatment with cytokinin-only, then supplementation with auxin at the beginning of the sampling period, on (a) cell number density and (b) nuclear DNA content determined at the beginning of the sampling period. Cells had previously been cultured for 8 days without added phytohormone in a hormone depletion regime. Cells entering the 8 day depletion period had previously been grown in standard CSV medium containing 9 μM 2,4-D and 0.23 μM kinetin. In this medium they were diluted at 4 day intervals from final cell density of about 6×10^6 per ml to one sixth of that density. At the beginning of the 8 day hormone depletion period were washed twice by centrifugation in medium without added hormone then resuspended in this medium to the standard initial culture density of 1×10^6 cells per ml. After 4 days they were again given the standard dilution to one sixth in the same hormone-free medium but without centrifugation. After the 8 day hormone depletion treatment cells were spun down and resuspended in CSV medium containing 0.23 μM kinetin and were cultured for 2 days, and then supplemented with final 9 μM of 2,4-D at time zero in the sampling period.

Fig. 6.4.

(a)


Cell number density
(cells / ml x10⁻⁴)
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10
8
6
4
2
0
0
12
18
24
30
42
Time (h)

(b)

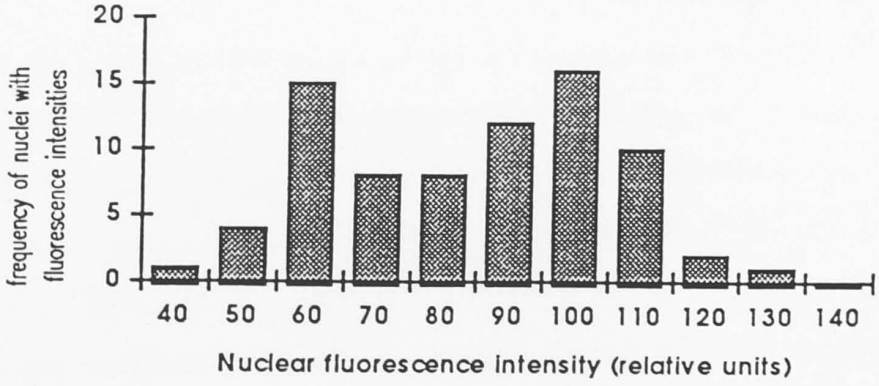

frequency of nuclei with
fluorescence intensities
20
15
10
5
0
40
50
60
70
80
90
100
110
120
130
140
Nuclear fluorescence intensity (relative units)

Fig. 6.5. Cells shown in Fig.6.4. which were used to test the effect of 2 day treatment with cytokinin then supplementation with auxin at the start of the sampling period, were also sampled for (a) content of $p34^{cdc2}$-like protein and (b) affinity purified $p34^{cdc2}$-like H1 kinase activity.

For estimation of $p34^{cdc2}$-like protein (PSTAIR protein) in the cell suspension culture, equal loadings of 50 µg protein were separated on 10-15% linear gradient acrylamide gel. Transferred proteins were probed with affinity-purified polyclonal anti-EGVPSTAIREISLLKE antibody and bound antibody was detected by ^{125}I anti-rabbit IgG. The image shown obtained by exposure in a phosphorImager and also was analysed to determine levels of $p34^{cdc2}$-like protein by quantification of bound isotope shown above the image.

For measurement of the activity of $p34^{cdc2}$-like protein kinase, 0.1g of fresh weight of cells ground in liquid nitrogen were extracted in NDE buffer (described in general methods), purified by 20 µl of $p13^{suc1}$ beads and eluted with 50 µl of 0.5 mg/ml of free $p13^{suc1}$. The activity of $p34^{cdc2}$-like protein kinase was measured using Histone H1 as substrate at 30°C for 5 min. The amount of ^{32}P transferred was measured by placing 20 µl of reaction mixture on P81 phosphocellulose paper washing with phosphoric acid then counting in a scintillation counter. The radioactivity of labelled Histone H1, shown at the bottom of the figure, was obtained by separation 30 µl of reaction mixture on a 12% acrylamide gel and then exposure in a phosphorImager.

Fig. 6.5.

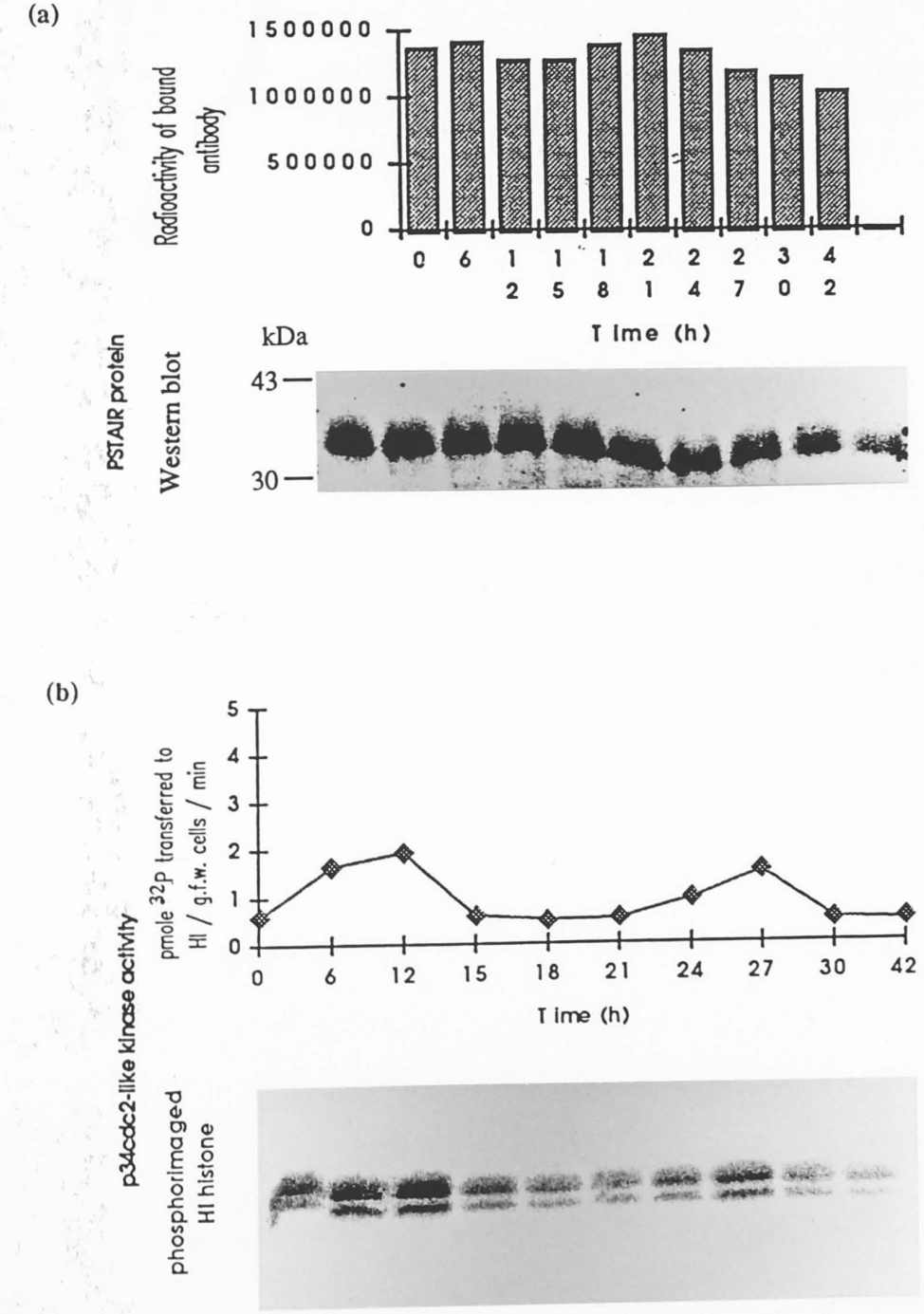
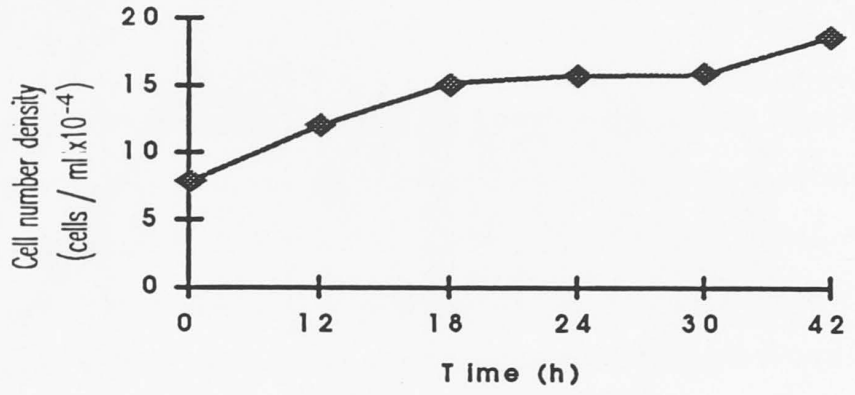

(a)
1500000
1000000
500000
0
Radioactivity of bound antibody
0 6 12 15 18 21 24 27 30 42
Time (h)
kDa
43
30
PSTAIR protein
Western blot
(b)
5
4
3
2
1
0
pmole 32P transferred to HI / g.f.w. cells / min
0 6 12 15 18 21 24 27 30 42
Time (h)
p34cdc2-like kinase activity
phosphorimaged HI histone

Fig. 6.6. Effect of 2 day treatment with auxin-only, then supplementation with cytokinin at the beginning of the sampling period, on (a) cell number density and (b) nuclear DNA content determined at the beginning of the sampling period. Cells had previously been cultured for 8 days without added phytohormone in a hormone depletion regime. Cells entering the 8 day depletion period had previously been grown in standard CSV medium containing 9 μM 2,4-D and 0.23 μM kinetin. In this medium they were diluted at 4 day intervals from final cell density of about 6 x 10^6 per ml to one sixth of that density. At the beginning the 8 day hormone depletion period were washed twice by centrifugation in medium without added hormone then resuspended in this medium to the standard initial culture density of 1 x 10^6 cells per ml. After 4 days they were again given the standard dilution to one sixth in the same hormone free medium but without centrifugation. After 8 day hormone depletion treatment cell were spun down and resuspended in CSV medium containing 9 μM of 2,4-D and were cultured for 2 days, and then supplemented with final 0.23 μM of kinetin at time zero in the sampling period.

Fig. 6.6.

(a)


Cell number density
(cells / ml x10-4)
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15
10
5
0
0
12
18
24
30
42
T ime (h)

(b)

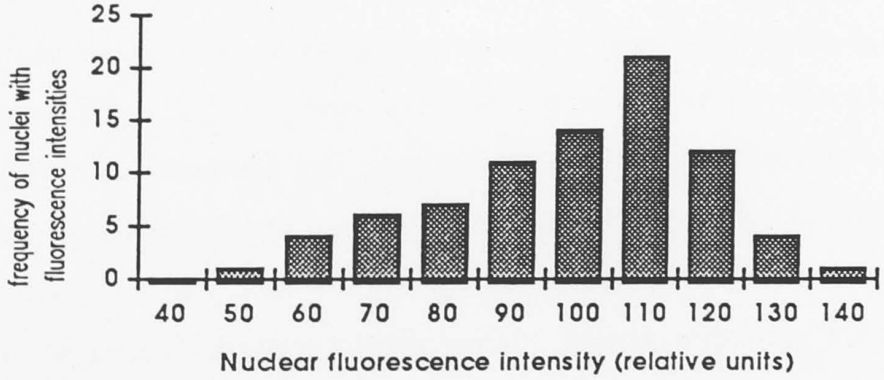

frequency of nuclei with
fluorescence intensities
25
20
15
10
5
0
40
50
60
70
80
90
100
110
120
130
140
Nuclear fluorescence intensity (relative units)

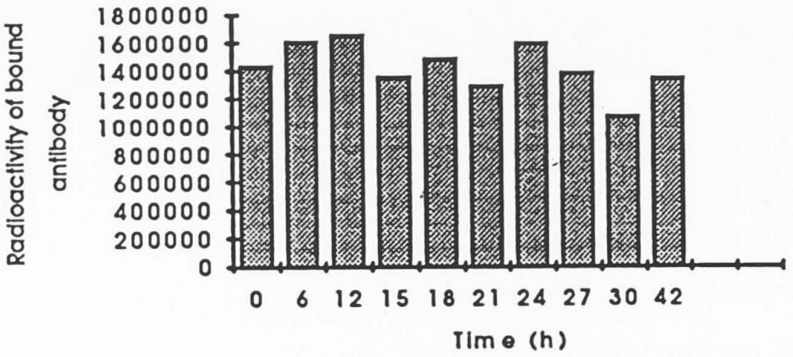
Fig. 6.7. Cells shown in Fig. 6.6 that were used to test the effect of resuspension in medium with auxin and supplement with cytokinin were also sampled for (a) content of $p34^{cdc2}$-like protein and (b) affinity purified $p34^{cdc2}$-like H1 kinase activity.

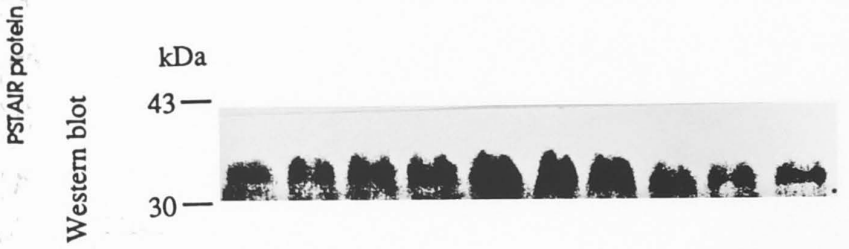
For estimation of $p34^{cdc2}$-like protein (PSTAIR protein) in the cells suspension culture, equal loadings of 50 µg protein were separated on 10-15% linear gradient acrylamide gel. Protein transferred for Western blotting were probed with affinity-purified polyclonal anti-EGVPSTAIREISLLKE antibody and bound antibody was detected by ^{125}I anti-rabbit IgG. The image shown was obtained by exposure in a phosphorImager and also was analysed to determine levels of $p34^{cdc2}$-like protein by quantification of bound isotope, shown above the image.

For measurement of the activity of $p34^{cdc2}$-like protein kinase, 0.1g fresh weight of cells ground in liquid nitrogen were extracted in NDE buffer (described in general methods), purified by 20 µl of $p13^{suc1}$ beads and eluted with 50 µl of 0.5 mg/ml of free $p13^{suc1}$. The activity of $p34^{cdc2}$-like protein kinase was measured using Histone H1 as substrate at 30°C for 5 min. The amount of ^{32}P transferred was measured by placing 20 µl of reaction mixture on P81 phosphocellulose paper washing with phosphoric acid then counting in a scintillation counter. The radioactivity of labelled Histone H1, shown at the bottom of the figure, was obtained by separation 30 µl of reaction mixture on a 12% acrylamide gel and then exposure in a phosphorImager.

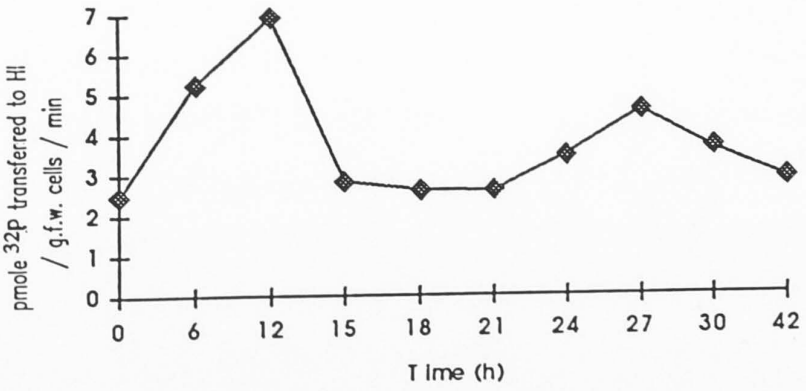
Fig. 6.7.

(a)


Radioactivity of bound antibody
1800000
1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
0 6 12 15 18 21 24 27 30 42
Time (h)


PSTAIR protein
Western blot
kDa
43
30

(b)


pmole 32p transferred to HI / g.f.w. cells / min
7
6
5
4
3
2
1
0
0 6 12 15 18 21 24 27 30 42
Time (h)

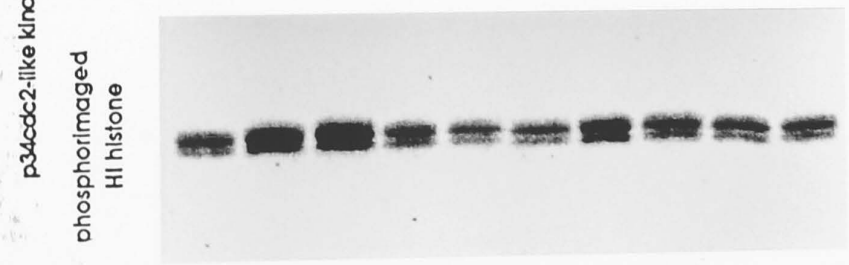
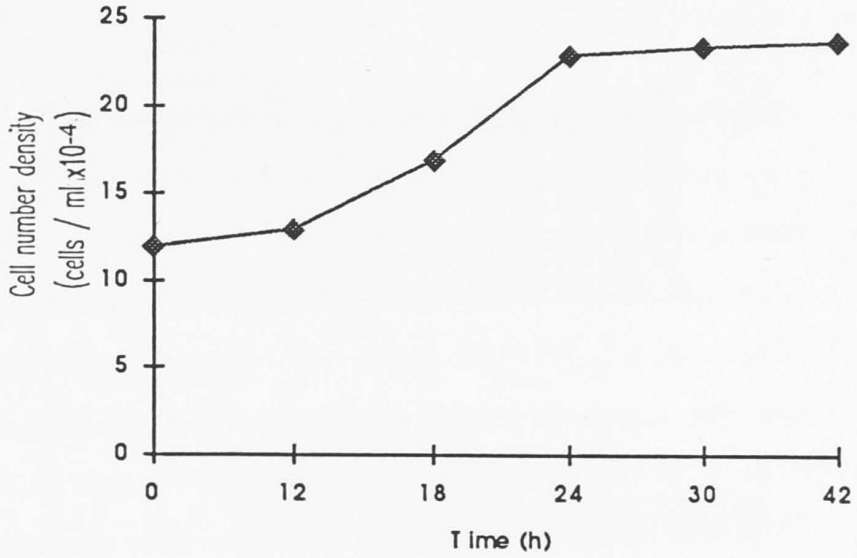

p34cdc2-like kinase activity
phosphorImaged HI histone

Fig. 6.8. Effect 2 day pre-treatment, with both auxin and cytokinin then continued incubation in the same medium during the sampling period, on (a) cell number density and (b) nuclear DNA content measured at the beginning of the sampling period. Cell had previously been cultured for 8 days without added phytohormone in a hormone depletion regime. Cells entering the 8 day depletion period had previously been grown in standard CSV medium containing 9 μM 2,4-D and 0.23 μM kinetin. In this medium they were diluted at 4 day intervals from final cell density of about 6 x 10^6 per ml to one sixth of that density. At the beginning of the 8 day hormone depletion period were washed twice by centrifugation in medium without added hormone then resuspended in this medium to the standard initial culture density of 1 x 10^6 cells per ml. After 4 days they were again given the standard dilution to one sixth in the same hormone-free medium but without centrifugation. After the 8 day hormone depletion treatment cells were spun down and were resuspended in complete CSV medium containing 9 μM of 2,4-D and 0.23 μM of kinetin and were cultured for 2 days, and then a sampling period was commenced that continued for 42h.

Fig. 6.8.

(a)


Cell number density
(cells / ml x10-4)
25
20
15
10
5
0
0
12
18
24
30
42
Time (h)

(b)

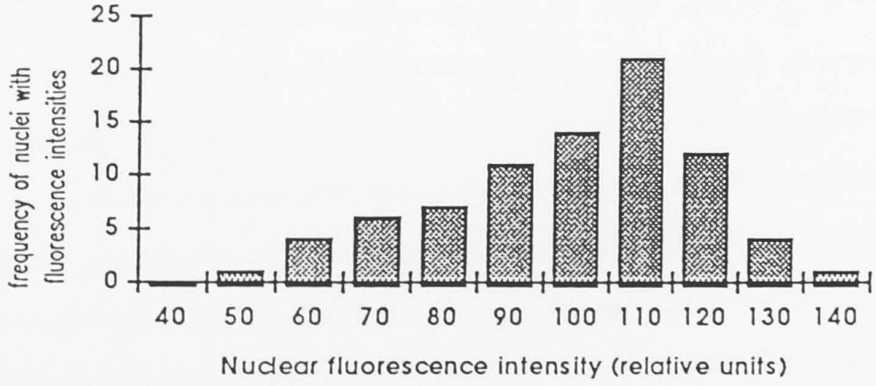

frequency of nuclei with
fluorescence intensities
25
20
15
10
5
0
40
50
60
70
80
90
100
110
120
130
140
Nuclear fluorescence intensity (relative units)

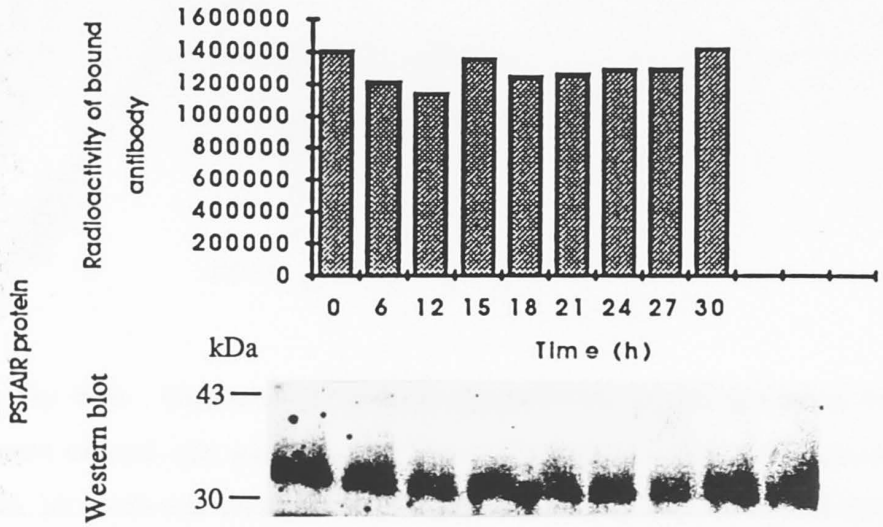
Fig. 6.9. Cells shown in Fig.6.8 that were used to test the effect of resuspension in medium with both auxin and cytokinin were also sampled for (a) content of $p34^{cdc2}$-like protein and (b) affinity purified $p34^{cdc2}$-like H1 kinase activity.

For estimation of $p34^{cdc2}$-like protein (PSTAIR protein) in the suspension culture, equal loadings of 50 µg protein were separated on 10-15% linear gradient acrylamide gel. Transferred proteins were probed with affinity-purified polyclonal anti-EGVPSTAIREISLLKE antibody and bound antibody was detected by ^{125}I anti-rabbit IgG. The image shown was obtained by exposure in a phosphorImager and also was analysed to determine levels of $p34^{cdc2}$-like protein by quantification of bound isotope, shown above the image.

For measurement of the activity of $p34^{cdc2}$-like protein kinase, 0.1g fresh weight of cells ground in liquid nitrogen were extracted in NDE buffer (described in general methods), purified by 20 µl of $p13^{suc1}$ beads and eluted with 50 µl of 0.5 mg/ml of free $p13^{suc1}$. The activity of $p34^{cdc2}$-like protein kinase was measured using Histone H1 as substrate at 30°C for 5 min. The amount of ^{32}P transferred was measured by placing 20 µl of reaction mixture on P81 phosphocellulose paper washing with phosphoric acid then counting in a scintillation counter. The radioactivity of labelled Histone H1, shown at the bottom of the figure, was obtained by separation 30 µl of reaction mixture on a 12% acrylamide gel and then exposure in a phosphorImager.

Fig. 6.9.

(a)


PSTAIR protein
Radioactivity of bound antibody
1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
0 6 12 15 18 21 24 27 30
Time (h)
Western blot
kDa
43
30

(b)

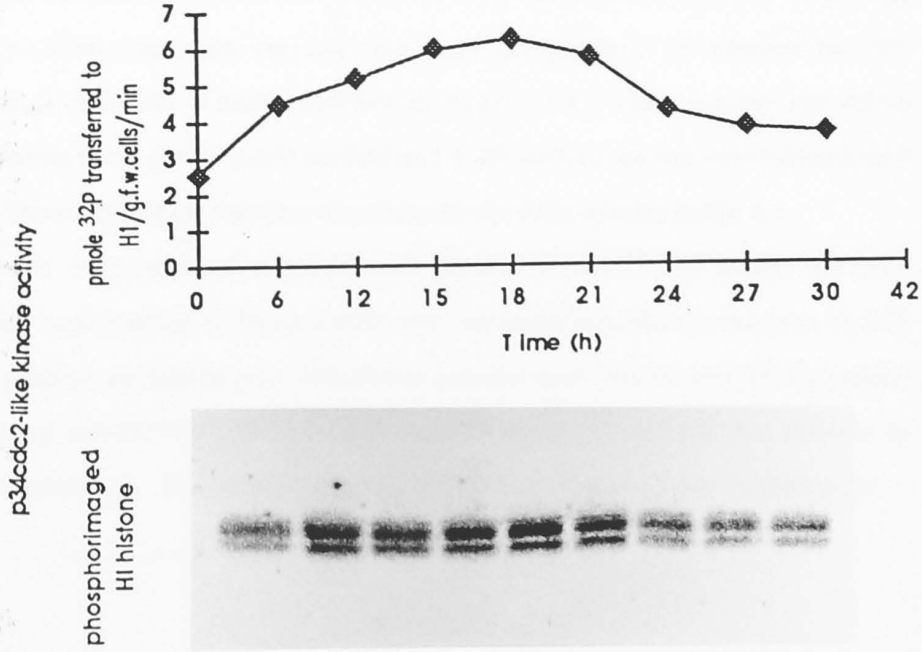

p34cdc2-like kinase activity
pmole 32p transferred to H1/g.f.w.cells/min
7
6
5
4
3
2
1
0
0 6 12 15 18 21 24 27 30 42
Time (h)
phosphorimaged HI histone

Fig. 6.10.

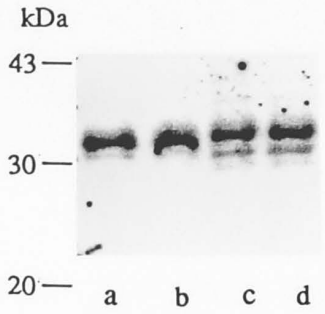


Fig. 6.10. Electrophoresis patterns of $p34^{cdc2}$-like protein in extracts from suspension cultured cells taken from (a) hormone-free CSV medium, (b) kinetin-only medium, (c) 2,4-D-only medium and (d) medium containing both kinetin and 2,4-D. Cells had previously been cultured for 8 days without added phytohormone by centrifugation in medium without added hormone then resuspended in this medium to the standard initial culture density of 1×10^6 cells per ml. After 4 days they were again given the standard dilution to one sixth in the same hormone free medium. After 8 day depletion of hormone, cells were spun down, and resuspended in (a) hormone-free CSV medium; (b) 0.23 μM of kinetin medium; (c) 9 μM of 2,4-D medium or (d) both 0.23 μM of kinetin and 9 μM of 2,4-D medium at 5×10^5 cells per ml and were cultured for 4 days. These samples are therefore identical with the 4 day samples in Fig. 6.1.

For observation of electrophoresis patterns of $p34^{cdc2}$-like protein (PSTAIR protein), equal loadings of 50 μg protein were separated in parallel on the same 10-15% linear gradient acrylamide gel. Transferred proteins were probed with affinity-purified poly clonal anti-EGVPSTAIREISLLKE antibody and bound antibody was detected by ^{125}I anti rabbit IgG. The image shown was obtained by exposure in a phosphorImager.

Fig. 6.11. *In vitro* activation of $p34^{cdc2}$-like H1 histone kinase activity by potato acid phosphatase treatment of $p34^{cdc2}$-like protein purified by $p13^{suc1}$ affinity from extracts of suspension cultured cells treated by deprivation of either auxin or cytokinin.

Cells from which the enzyme was derived were identical with those present at time 0h in Figs. 6.5, 6.7. The enzyme K is from cells treated with kinetin-only and taken at time 0h of 42h sampling period (Fig. 6.5) and D is from cells treated with 2,4-D-only and taken at 0h of 42h sampling period (Fig. 6.7).

K-P or D-P indicates acid phosphatase treatment of the enzyme K or D by incubation for 3 or 5 min at 30°C with 1 unit of acid phosphatase which was dissolved in phosphatase buffer consisting of 50 mM PIPES (pH 6.0), 0.1% β-mercaptoethanol, 0.1 mM PMSF, 0.1 mM benzamidine, 10 μg/ml soybean trypsin inhibitor and 10 μg/ml leupeptin.

K-C or D-C are control incubation of enzyme K or D with phosphatase buffer lacking only phosphatase enzyme.

Fig. 6.11.

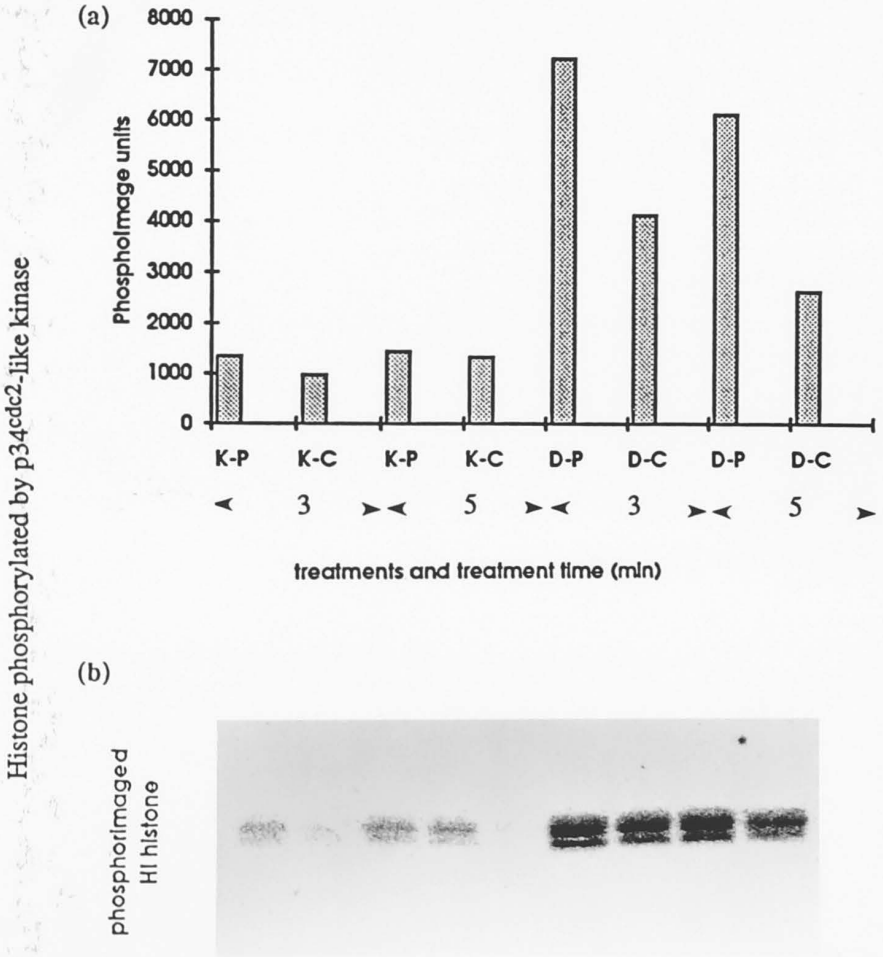

(a)
8000
7000
6000
5000
4000
3000
2000
1000
0
Phospholmage units
K-P
K-C
K-P
K-C
D-P
D-C
D-P
D-C
3
5
3
5
treatments and treatment time (min)
Histone phosphorylated by p34cdc2-like kinase
(b)
phosphorlmaged
HI hlstone

Fig. 6.12. Effects of auxin and cytokinin on the growth of cell suspension culture of *N. plumbaginifolia:* This figure shows the appearance of the four cultures described in Fig. 6.1 at the mid point of the sampling period (arrowed in Fig. 6.1) prior to the supplementation of each culture so that each subsequently contained 2,4-D and kinetin. In the 4 day period prior to photographing the cultures were incubated in; B, hormone-free medium, ie curve 1 in Fig. 6.1; C, medium with kinetin only, ie curve 2 in Fig. 6.1; A, medium with 2,4-D only, ie curve 3 in Fig. 6.1; D, medium with both kinetin and 2,4-D. Cells entering the 4 day hormone treatments had previously been washed twice then suspended in CSV 0 medium at 1 x 10^6 cells per ml for 4 days and then diluted in CSV 0 medium to 1/6 and were cultured for another 4 days, then spun down and resuspended in the different hormone mixtures.

Fig. 6.13. Dedifferentiation of stem pith of tobacco Wisconsin 38 induced by auxin and cytokinin: pith tissue was cultivated for 4 days; top left on hormone free solid MS medium; top right on solid MS medium containing 0.56 μM of BAP; bottom left on solid MS medium containing 0.56 μM of BAP and 5.4 μM of NAA and bottom right on solid MS medium containing 5.4 μM of NAA. All of the cultures were kept in an incubator at 25°C in with 8 hour light and 16 hour darkness.

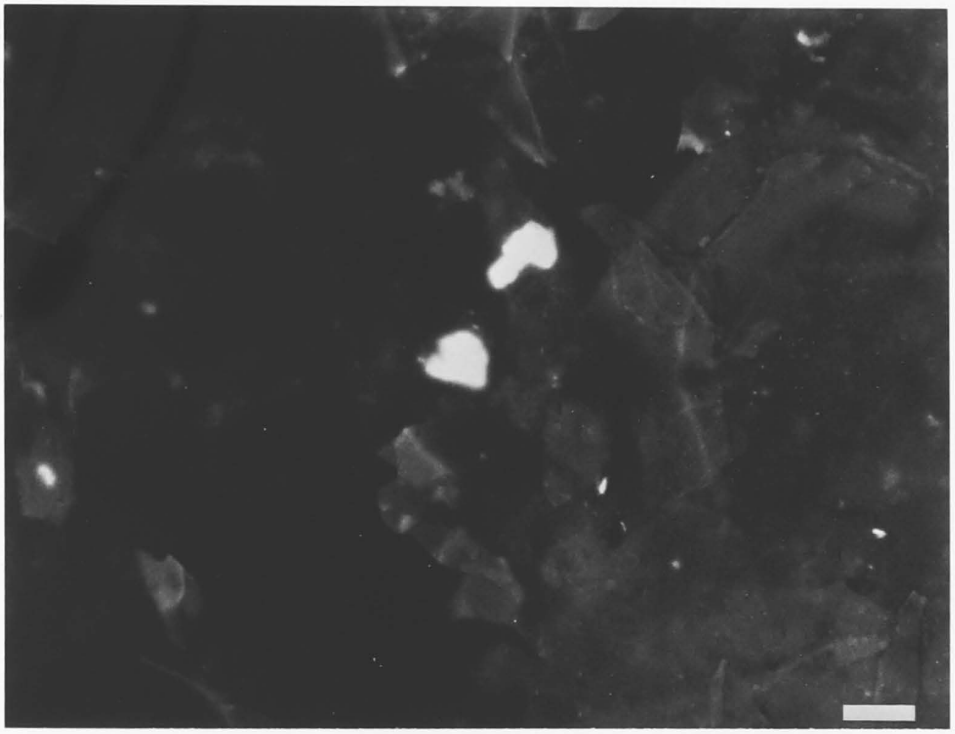
Fig. 6.12.

Fig. 6.13.

Fig. 6.14. Attempted incorporation of BrdU into nuclear DNA in pith tissue of tobacco Wisconsin 38 incubated on hormone-free solid MS medium. (a) presence of nuclei detectable by DAPI stain, seen as brightly-fluorescing and irregular in outline; (b) absence of BrdU incorporation in same field of view. Positive incorporation of BrdU, when present is seen as bright staining of in nuclei due to fluorescent antibody binding, as can be seen in Fig. 6.17.b. For assay of BrdU incorporation sub-segments were cut from pith tissue segments that had been incubated on the agar for 8 days and the subsegments were incubated in liquid medium containing the same hormone and with 100 μM of BrdU for 15 hours, cryo-sectioned at 8 μm thickness, probed with antibody against BrdU-DNA and stained with DAPI. Scale bar, 10 μm.

Fig. 6.14.

(a)



(b)

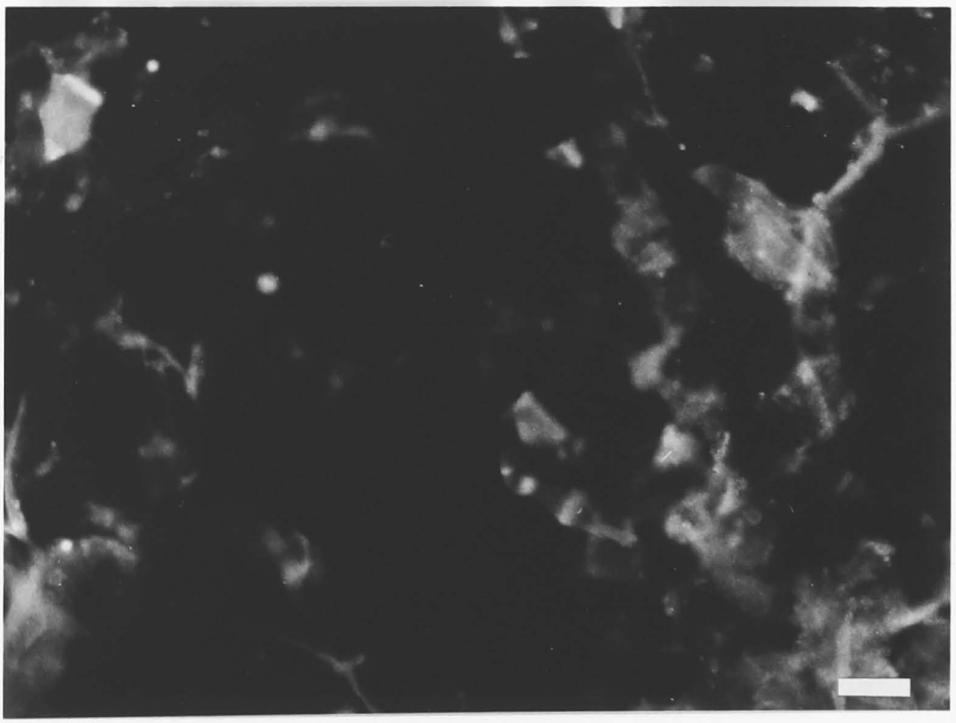
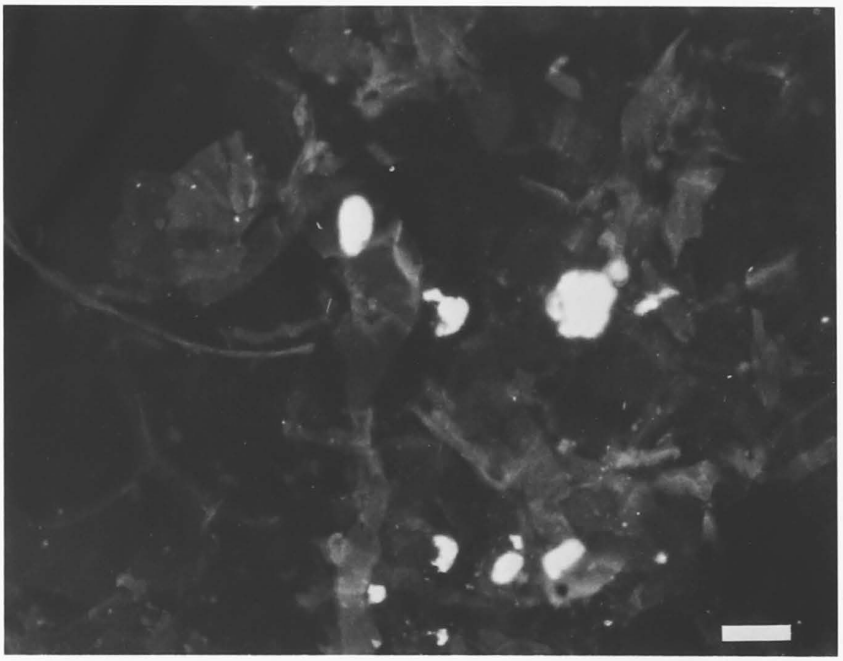


Fig. 6.15 Attempted incorporation of BrdU into nuclear DNA in pith tissue of tobacco Wisconsin 38 incubated on BAP-only solid MS medium. (a) presence of nuclei detectable by DAPI stain, seen as brightly-fluorescing and irregular in outline; (b) absence of BrdU incorporation in same field of view. Positive incorporation of BrdU, when present is seen as bright staining in nuclei due to fluorescent antibody binding, as can be seen in Fig. 6.17.b. For assay of BrdU incorporation sub-segments were cut from pith tissue segments that had been incubated on the agar for 8 days and the subsegments were incubated in liquid medium containing the same hormone and with 100 μM of BrdU for 15 hours, cryo-sectioned at 8 μm thickness, probed with antibody against BrdU-DNA and stained with DAPI. Scale bar, 10 μm.

Fig. 6.15.

(a)



(b)

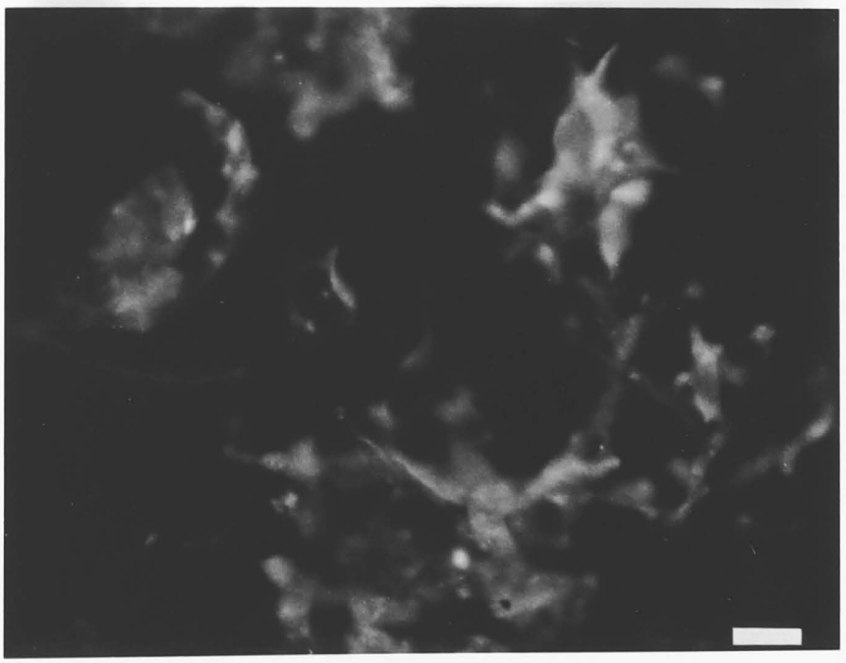
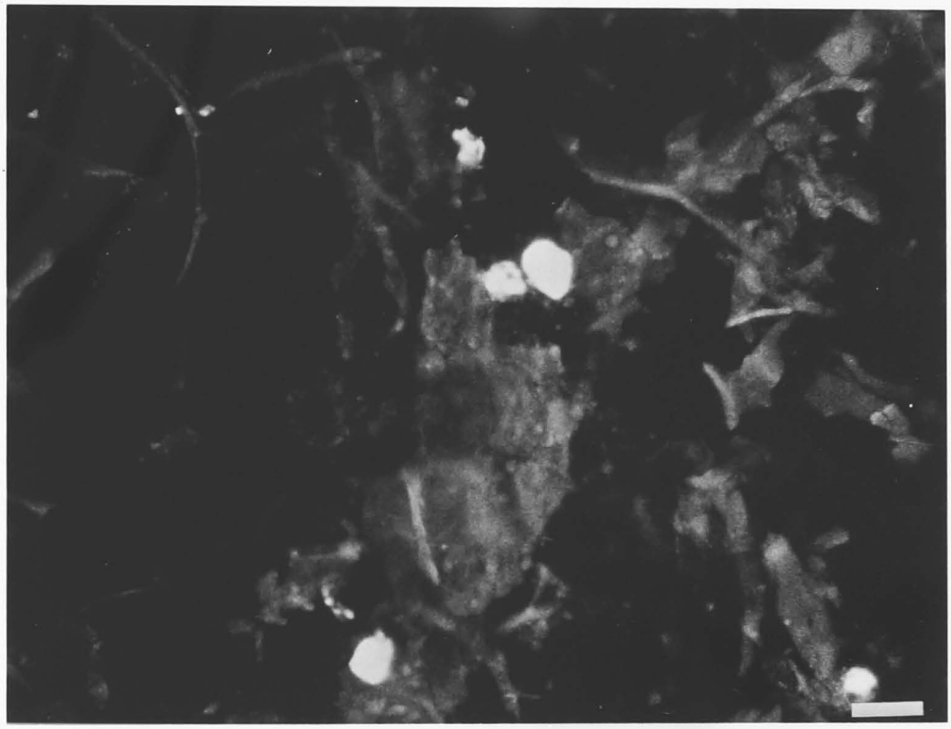


Fig. 6.16 Attempted incorporation of BrdU into nuclear DNA in pith tissue of tobacco Wisconsin 38 incubated on NAA-only solid MS medium. (a) presence of nuclei detectable by DAPI stain, seen as brightly-fluorescing and irregular in outline; (b) absence of BrdU incorporation in same field of view. Positive incorporation of BrdU, when present is seen as bright staining in nuclei due to fluorescent antibody binding, as can be seen in Fig. 6.17.b. For assay of BrdU incorporation sub-segments were cut from pith tissue segments that had been incubated on the agar for 8 days and the subsegments were incubated in liquid medium containing the same hormone and with 100 μM of BrdU for 15 hours, cryo-sectioned at 8 μm thickness, probed with antibody against BrdU-DNA and stained with DAPI. Scale bar, 10 μm.

Fig. 6.16.

(a



(b

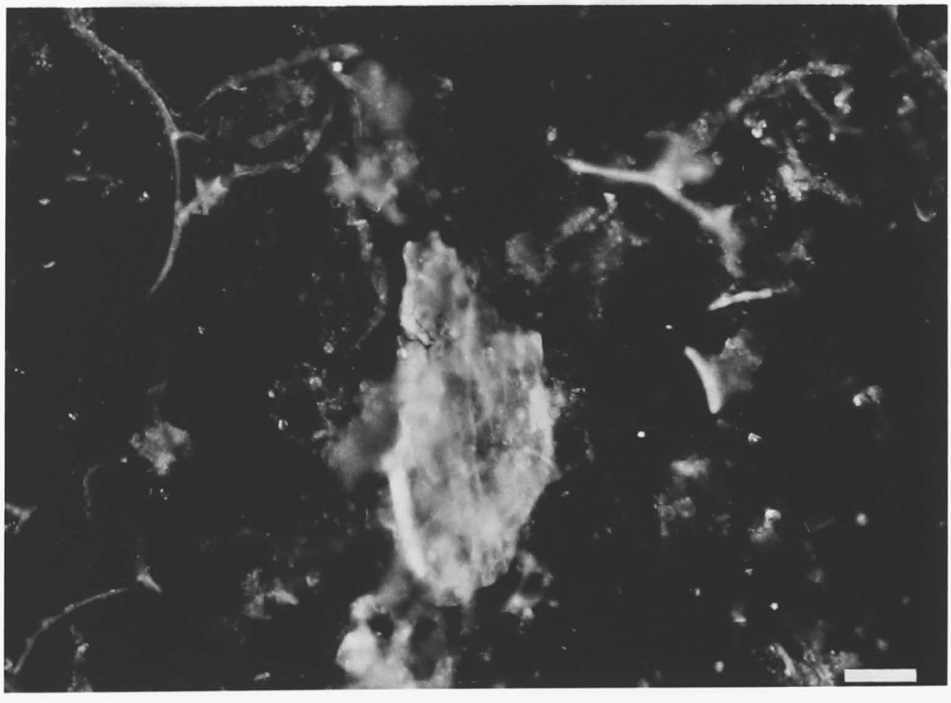
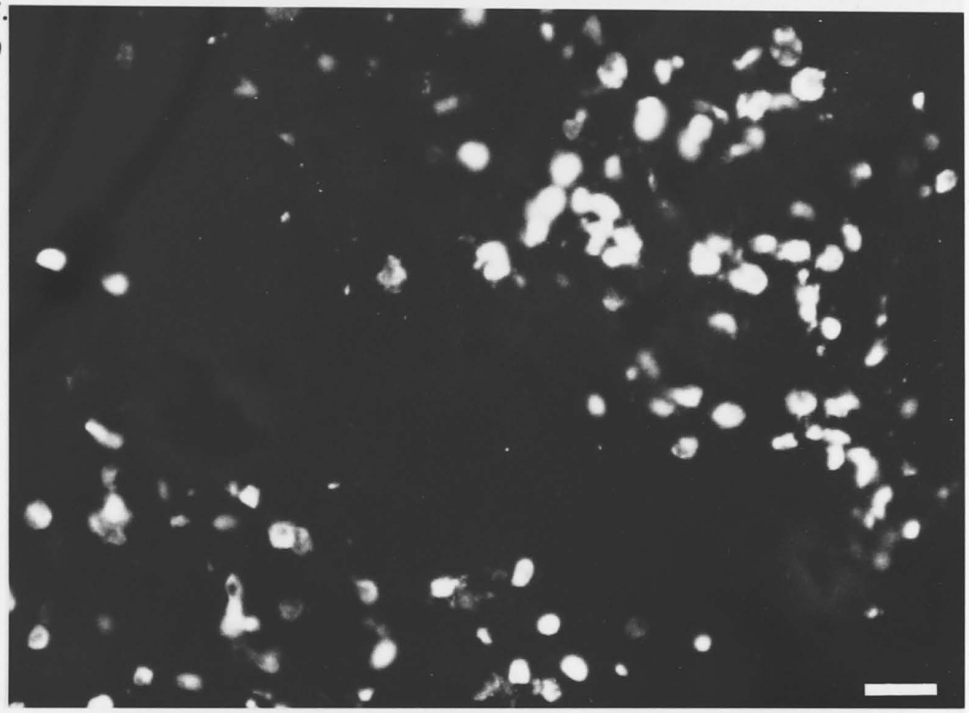


Fig. 6.17 Attempted incorporation of BrdU into nuclear DNA in pith tissue of tobacco Wisconsin 38 incubated on NAA and BAP both solid MS medium. (a) presence of nuclei detectable by DAPI stain, seen as brightly-fluorescing and irregular in outline; (b) presence of BrdU incorporation in same field of view. For assay of BrdU incorporation sub-segments were cut from pith tissue segments that had been incubated on the agar for 8 days and the subsegments were incubated in liquid medium containing the same hormone mixture and with 100 μM of BrdU for 15 hours, crys-sectioned at 8 μ m thickness, probed with antibody against BrdU-DNA and stained with DAPI. Scale bar, 10 μm.

Fig. 6.17.

(a)



(b)

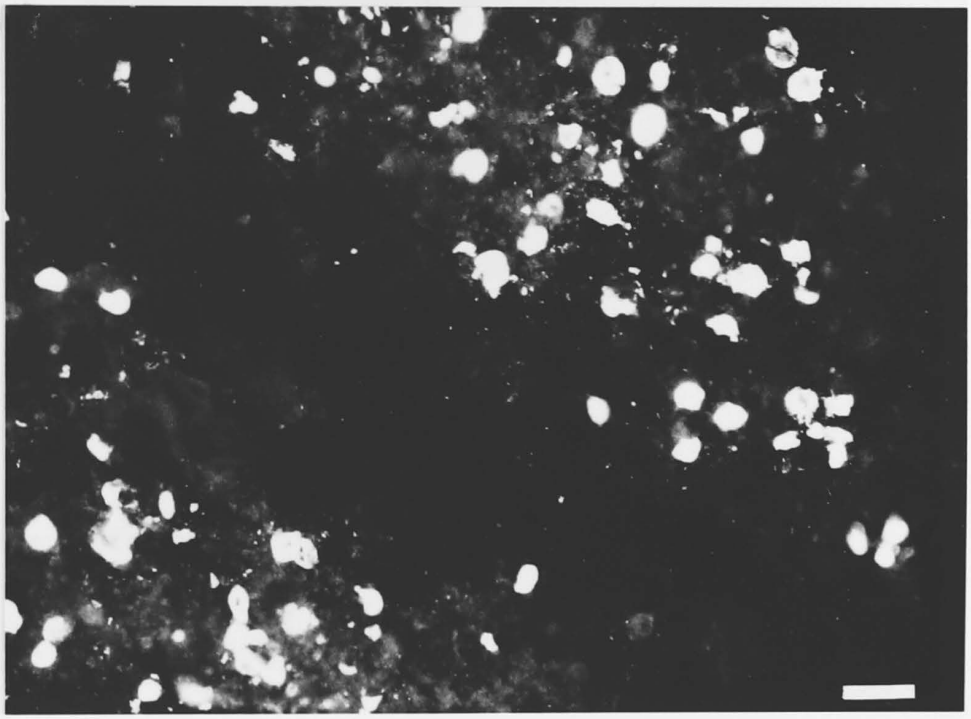


Fig. 6.18.

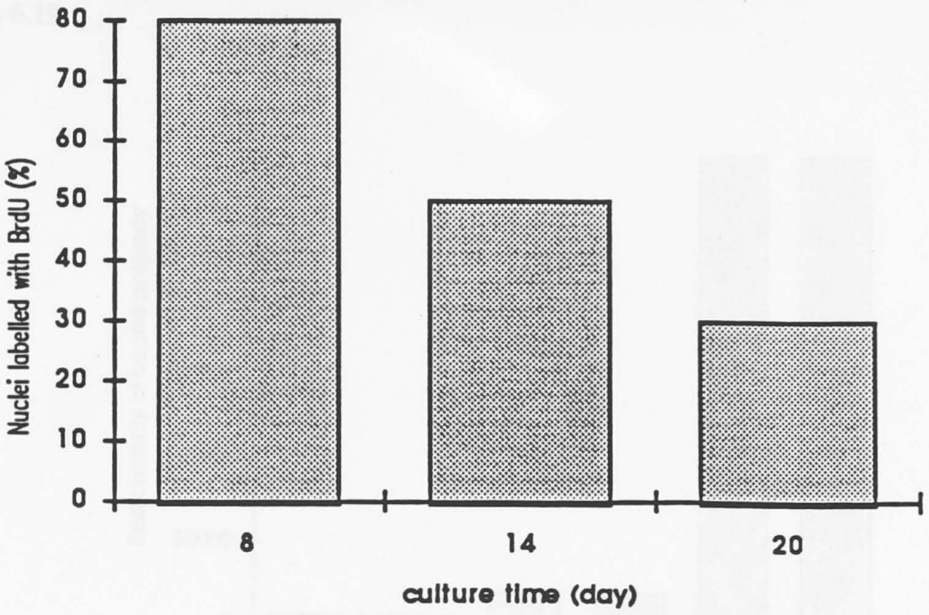


Fig. 6.18. Frequency of cells incorporating BrdU into nuclear DNA in tobacco stem pith tissue cultivated on solid MS medium containing 5.4 μM of NAA and 0.56 μM of BAP, sampled at day 8, day 14 and day 20, subdivided in 5 mm x 2 mm x 2 mm pieces and labelled with 100 μM of BrdU for 15 hours in the liquid medium containing the same hormone mixture. 8 μM thick sections cut from each sample were probed with antibody against BrdU-DNA and stained with DAPI. The percentage of nuclei labelled with BrdU was obtained by ratio with nuclear number determined in the same section by DAPI staining.

Fig. 6.19.

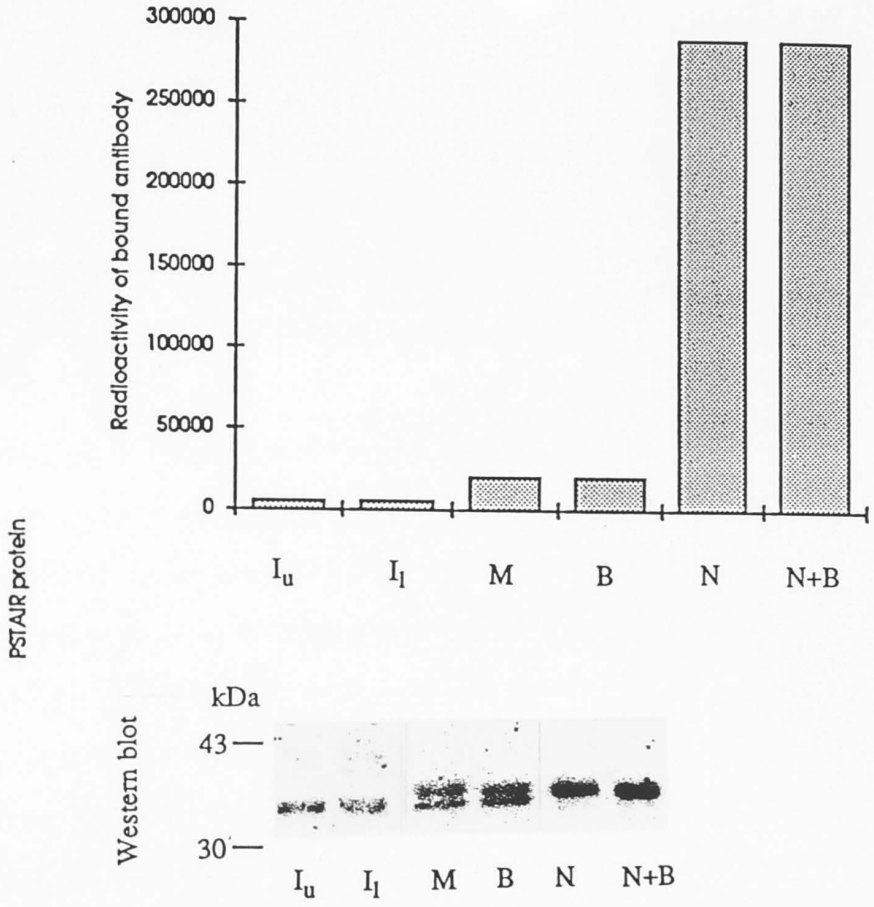


Fig. 6.19. Levels of p34^{cdc2}-like protein in stem pith at time of excision from the intact plant (I), compared with level established after 14 day incubation on; MS medium without added hormone (M); medium with 0.56 μM BAP (B); with 5.4 μM NAA (N); or with 0.56 μM BAP and 5.4 μM NAA (N + B). About 30 segments comprised each sample and equal loadings of 70 μg extracted total protein after concentration by acetone precipitation, were separated on 10-15% linear gradient acrylamide gels. Transferred proteins were probed with a single batch of affinity-purified polyclonal anti-EGVPSTAIREISLLKE antibody and bound antibody was detected by ^{125}I anti-rabbit IgG. The image shown was obtained by exposure in a phosphorImager and was analysed to determine levels of p34^{cdc2}-like protein by quantification of bound isotope.

I_u shows protein from an upper region of stem 10-15 cm below the apex and I_l from a lower region 10-15 cm above the base.

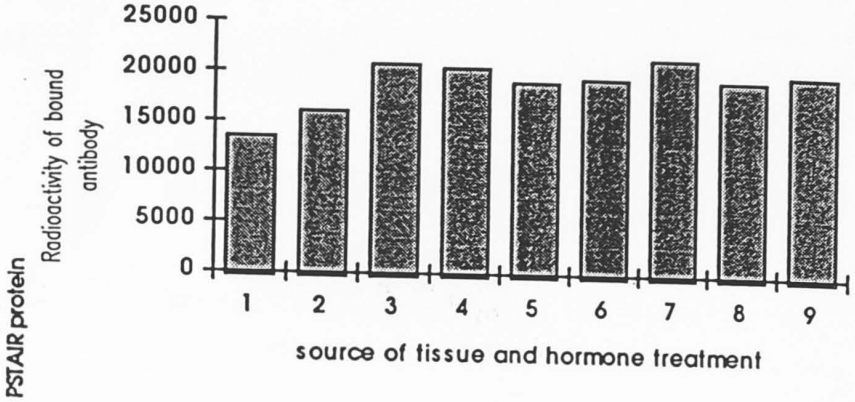
Fig. 6.20. Levels of p34^{cdc2}-like protein (PSTAIR protein) in the stem pith taken from 60 cm tall plants of tobacco Wisconsin 38 and after excision cultivated on solid MS media containing different levels of auxin (NAA) and cytokinin (BAP) for 14 days. About 30 segments comprised each sample and equal loadings of 70 µg extracted total protein after concentration by acetone precipitation, were separated on 10-15% linear gradient acrylamide gels. Transferred proteins were probed simultaneously with a single batch of affinity-purified polyclonal anti-EGVPSTAIREISLLKE antibody and bound antibody was detected by ^{125}I anti-rabbit IgG. The image shown was obtained by exposure in a phosphorImager and was analysed to determine levels of p34^{cdc2}-like protein by quantification of bound isotope.

(a) shows a quantitative analysis of p34^{cdc2}-like protein level in pith incubated without added hormone or with cytokinin only. (b) shows the Western blot from which they derive. Note that the ordinate scale in (a) is 14 times smaller than in (c) and that in (b) a double band of p34^{cdc2}-like protein is resolved. In (a) and (b) treatments 1 to 6 involved transfer of pith onto MS 0, medium without added hormone, and treatment 7 to 9 transfer to medium with 0.56 µM BAP only. Origins of the pith from which the stem dedifferentiated, and in 1, 2 and 3 were 0-5 cm, 5-10 cm and 10-15 cm respectively below the stem apex while in 4, 5 and 6 were from lower in the stem, at 0-5 cm, 5-10 cm and 10-15 cm above the base. In 7, 8 and 9 the pith derived from 0-5 cm, 5-10 cm and 10-15 cm above the base.

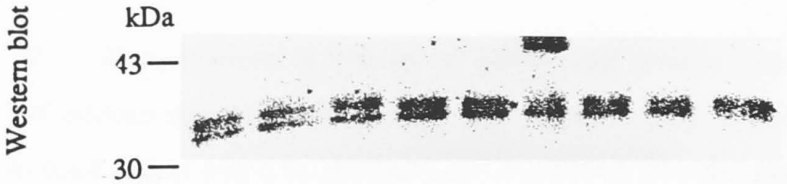
(c) and (d) show p34^{cdc2}-like protein levels in pith incubated with auxin only or with both auxin and BAP. A quantitative analysis is shown in (c) and the Western blot from which they derive in (d). In (c) treatment 1 to 6 involved incubation of pith on medium with 5.4 µM NAA only and treatment 7 to 9 incubation on medium with both 5.4 µM NAA and 0.56 µM BAP. Origins of the pith in 1, 2 and 3 were 0-5 cm, 5-10 cm and 10-15 cm respectively below the stem apex, while in 4, 5 and 6 were from lower in the stem at 0-5 cm, 5-10 cm and 10-15 cm respectively above the stem base. In 7, 8 and 9 the pith was excised from 0-5 cm, 5-10 cm and 10-15 cm above the base.

Fig. 6.20.

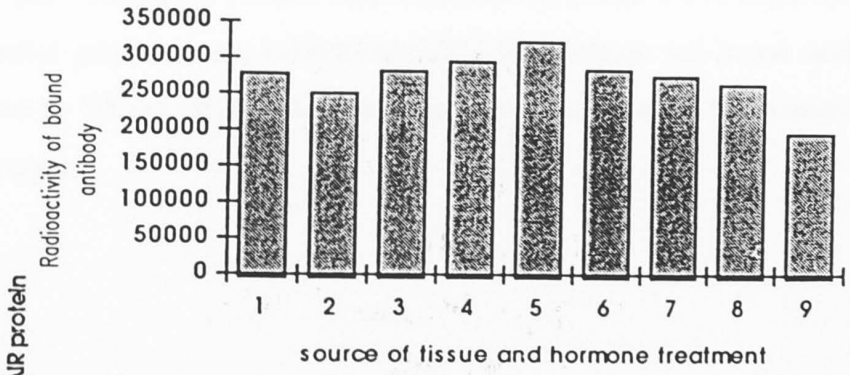
(a)


PSTAIR protein
Radioactivity of bound antibody
25000
20000
15000
10000
5000
0
1 2 3 4 5 6 7 8 9
source of tissue and hormone treatment

(b)


Western blot
kDa
43
30

(c)


PSTAIR protein
Radioactivity of bound antibody
350000
300000
250000
200000
150000
100000
50000
0
1 2 3 4 5 6 7 8 9
source of tissue and hormone treatment

(d)

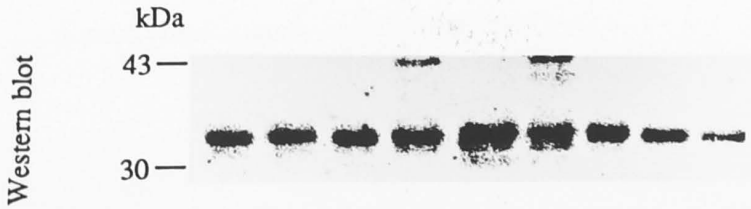

Western blot
kDa
43
30

Fig. 6.21.

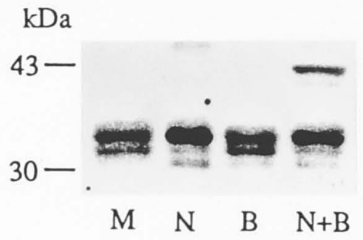


Fig. 6. 21. Electrophoresis in parallel of $p34^{cdc2}$-like proteins from pith incubated on; MS medium without added hormone (M); medium with 0.56 μM BAP (B); with 5.4 μM NAA (N); or with 0.56 μM BAP and 5.4 μM NAA (N + B). About 30 segments comprised each sample and equal loadings of 70 μg extracted total protein after concentration by acetone precipitation were separated on 10-15% linear gradient acrylamide gels. Transferred proteins were simultaneously probed with a single batch of affinity-purified polyclonal anti-EGVPSTAIREISLLKE antibody and bound antibody was detected by ^{125}I anti-rabbit IgG. The image shown was obtained by exposure in a phosphorImager.

Fig. 6.22. The relation between the recovered activity of $p34^{cdc2}$-like protein and the fresh weight of pith callus taken for extraction. The $p34^{cdc2}$-like protein kinase in 0.1g, 0.2g, 0.3g and 0.4g of pith callus which was cultivated on MS medium containing both 5.4 μM of NAA and 0.56 μM of BAP was purified by binding to 30 μl of $p13^{suc1}$ beads and was eluted with 50 μl of 0.5 mg/ml of free $p13^{suc1}$. The procedure for depletion of extract of agarose binding proteins and for detergent washing of $p34^{cdc2}$ bound to $p13^{suc1}$ beads is given in general methods. The activity of $p34^{cdc2}$-like protein kinase was measured using Histone H1 as substrate at 30°C during 5 min incubation. The amount of ^{32}P transferred was measured by placing 20 μl of reaction mixture on P81 phosphocellulose paper rinsing in phosphoric acid and by counting in a scintillation counter. The PhosphorImaged Histone H1 shown in the lower part of the Figure was obtained by separating 30 μl of reaction mixture on a 12% acrylamide gel and exposure in a phosphorImager.

Fig. 6.23. Comparison of the response of pith taken from the lower stem region and the upper stem region to mixture of auxin and cytokinin in terms of the appearance of $p34^{cdc2}$-like activity. Pith was taken from 0-15 cm below the stem apex (upper) and from 0-15 cm above the stem base (lower). Pith from the two sources was cultivated on solid MS medium containing both 5.4 μM of NAA and 0.56 μM of BAP (N+B), or 5.4 μ M of NAA only (N), or 0.56 μM of BAP (BAP) only, or on hormone-free solid MS medium (M). After 14 day incubation the $p34^{cdc2}$-like protein kinase in 0.25g of fresh weight of pith was purified with 30 μl of $p13^{suc1}$ beads and eluted with 50 μl of 0.5 mg/ml of free $p13^{suc1}$ as in Fig. 6.19. The procedure for depletion of extract of agarose binding proteins and for detergent washing of $p34^{cdc2}$ bound to $p13^{suc1}$ beads is given in general methods. The activity of $p34^{cdc2}$-like protein kinase was measured using Histone H1 as substrate at 30°C during 5 min incubation. The amount of ^{32}P transferred was measured by placing 20 μl of reaction mixture on P81 phosphocellulose paper rinsing in phosphoric acid and counting in a scintillation counter. The PhosphorImaged Histone H1 shown in the lower part of the Figure was obtained by separating 30 μl of reaction mixture on a 12% acrylamide gel and exposure in a phosphorImager.

Fig. 6.22.

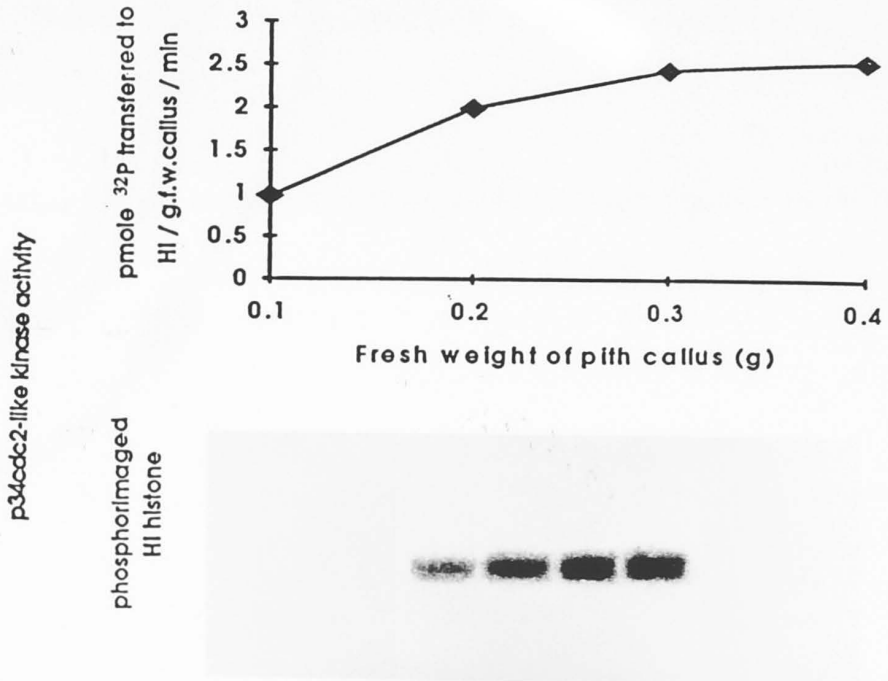


Fig. 6.23.

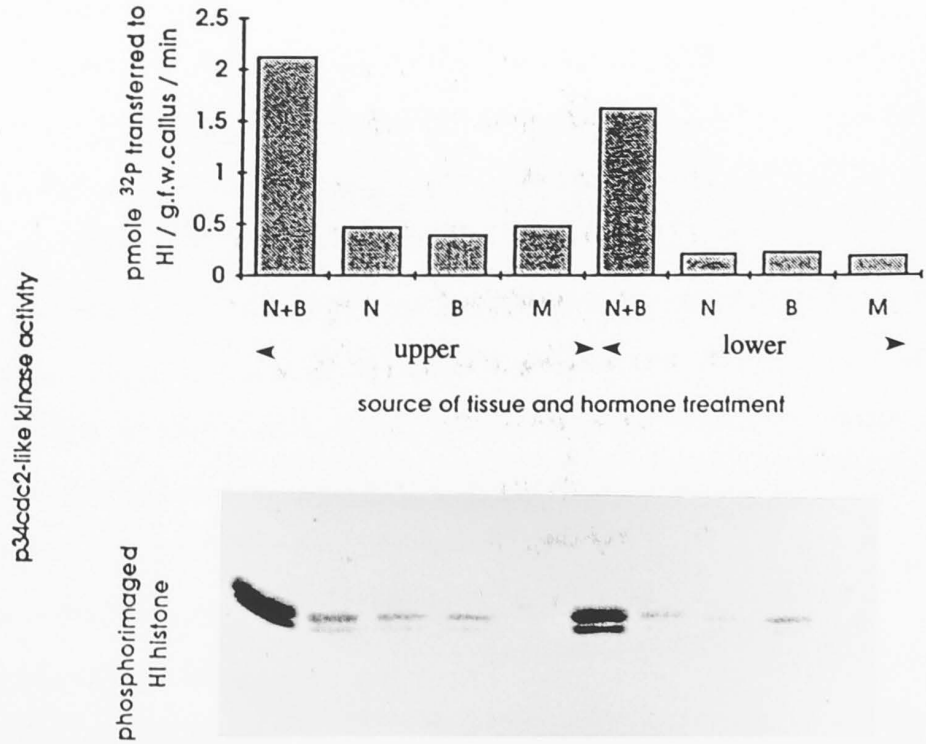


Fig. 6.24. Variability between individual plants in the response of their excised pith tissue to mixtures of auxin and cytokinin, in terms of the induced activity of $p34^{cdc2}$-like protein kinase. Pith taken from 0-15 cm above the stem base of plant 1, of plant 2 and of plant 3 was cultivated on; hormone-free solid MS medium (M); or medium containing 0.56 μM of (B); or medium containing 5.4 μM of NAA (N); or medium containing 5.4 μM of NAA and 0.56 μM of BAP (N+B). After 14 days the $p34^{cdc2}$-like protein kinase in 0.25g of fresh weight of treated pith tissue was purified by 30 μl of $p13^{suc1}$ beads and was eluted with 50 μl of 0.5 mg/ml of free $p13^{suc1}$. The procedure for depletion of extract of agarose binding proteins and for detergent washing of $p34^{cdc2}$ bound to $p13^{suc1}$ beads is given in general methods. The activity of $p34^{cdc2}$-like protein kinase was measured using Histone H1 as substrate at 30°C during 5 min incubation. The amount of ^{32}P transferred was measured by placing 20 μl of reaction mixture on P81 phosphocellulose paper rinsing in phosphoric acid and counting in a scintillation counter. The PhosphorImaged Histone H1 shown in the lower part of the Figure was obtained by separating 30 μl of reaction mixture on 12% acrylamide gel and exposure in a phosphorImager.

Fig. 6.25. Time course of change in activity of $p34^{cdc2}$-like protein kinase during dedifferentiation of tobacco pith induced by auxin and cytokinin. Pith taken from the same stem was incubated on; hormone-free solid MS medium (M); on medium containing 0.56 μM of BAP (B); on medium containing 5.4 μM of NAA (N); and on medium containing both 5.4 μM of NAA and 0.56 μM of BAP (N+B). The pith tissue for assay of $p34^{cdc2}$-like protein activity was taken at day 8, day 14 and day 20 of incubation. The procedure for depletion of extract of agarose binding proteins and for detergent washing of $p34^{cdc2}$ bound to $p13^{suc1}$ beads is given in general methods. The activity of $p34^{cdc2}$-like protein kinase was measured using Histone H1 as substrate at 30°C during 5 min incubation. The amount of ^{32}P transferred was measured by placing 20 μl of reaction mixture on P81 phosphocellulose paper rinsing in phosphoric acid and counting in a scintillation counter. The PhosphorImaged Histone H1 shown in the lower part of the Figure was obtained by separating 30 μl of reaction mixture on a 12% acrylamide gel and exposure in a phosphorImager.

Fig. 6.24.

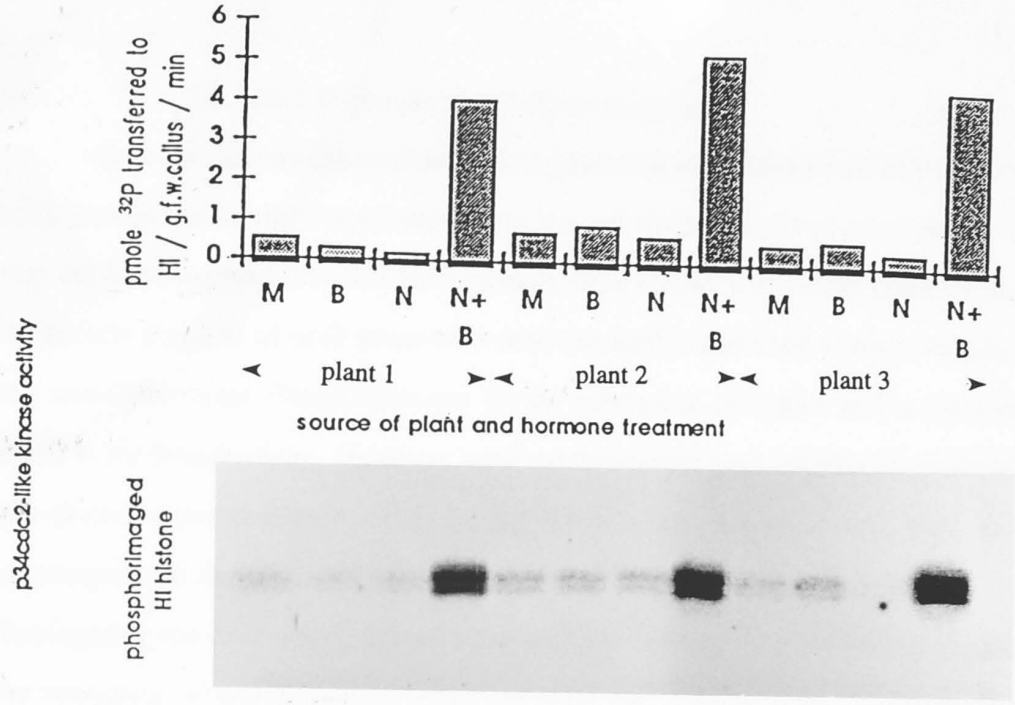
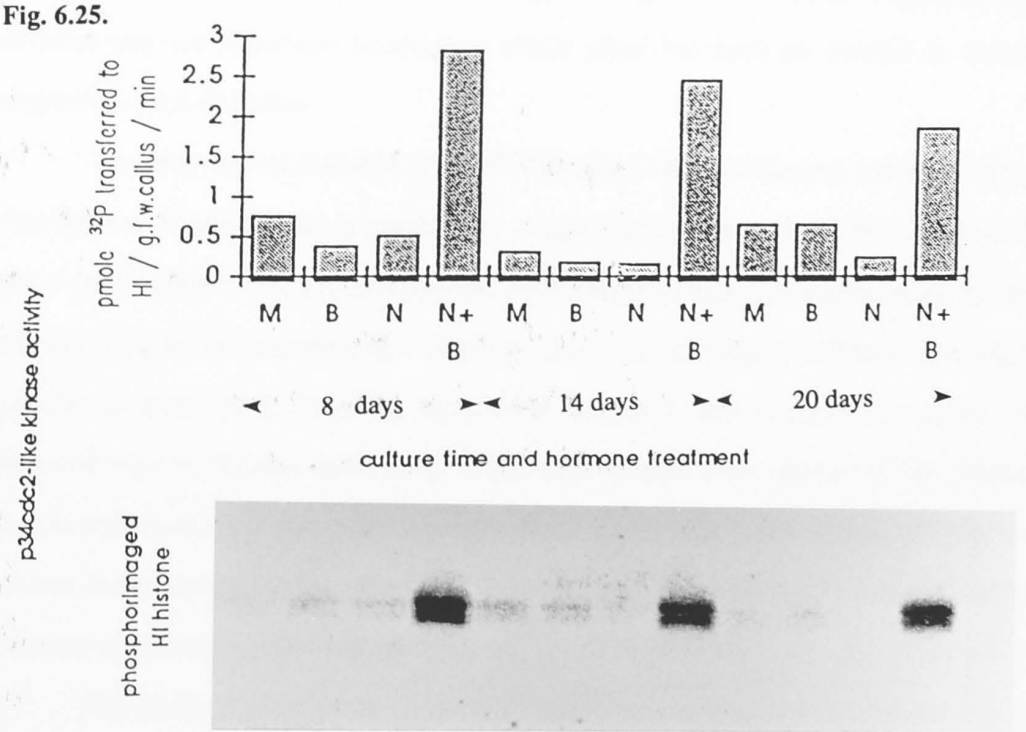


Fig. 6.25.



CHAPTER 7

GENERAL DISCUSSION

7.1. cdc2 in the plant cell division cycle

Genetical and biochemical analysis in yeast and animal cells has indicated that the *cdc2* gene is a universal control element in the cell division cycle of eukaryotes. Genes that are homologous with *cdc2* have recently been cloned from some plant species and the protein products of *cdc2* genes have been detected in plant cell extracts ranging from the unicellular plant *Chlamydomonas* to the meristems of higher plants (reviewed in detail in the Introduction). However, most studies to date have focused on establishing a one-to-one correspondence between cell cycle genes of yeast and their putative counterparts in the plant kingdom. The present study has extended this research by investigating the relationship between the catalytic activity of $p34^{cdc2}$-like enzyme and the occurence of specific events within the plant cell division cycle. In particular, new information has been obtained concerning developmental controls which act on cell division and are necessary to shape a whole plant but have no parallel in unicellular organisms such as yeasts.

To study the relationship of $p34^{cdc2}$ protein level and enzyme activity to progress through the division cycle in plant cells, suspension culture cells of *N. plumbaginifolia* were synchronised. To reveal potential perturbations from synchronousing procedures, which might be undetectable if a single method was employed, different synchronising treatments were used, including aphidicolin inhibition and nutrient starvation. Cells resumed the cell division cycle in G1 phase after release from starvation for nitrogen or for phosphorous, in S phase after release from aphidicolin block and in G2 phase after release from starvation for sucrose. Reassuringly, consistent evidence was obtained from the diverse methods of synchronising.

The level of $p34^{cdc2}$-like protein was held at a relatively constant ratio to other cell proteins throughout the cell cycle in suspension culture cells. The $p34^{cdc2}$-like protein in cells resuming from starvation for nitrogen did show a slow increase but this

did not correlate clearly with any cell cycle events and may present a restoration of level previously reduced by starvation. The finding of relatively constant levels of $p34^{cdc2}$-like protein in plant cells correlates with the evidence from proliferating yeast cells (Simanis and Nurse, 1986) and human cancer cells (Draetta and Beach, 1988). In no cell type has the rate of synthesis of $p34^{cdc2}$-like protein in the cell cycle been determined and it must be acknowledged that, from the measurements of total level made in this investigation, it is not possible to eliminate the occurence of minor fluctuations in rate of synthesis or degradation. Although significant changes in $p34^{cdc2}$-like protein level were not found in the synchronous cultures it should be noted that expression of the gene can be strongly regulated during normal development of multicellular organs, as observed in the wheat leaf (John et al., 1990) and carrot cotyledon (Gorst et al., 1991) and shown here following the application of phytohormones to plant tissues. Chapter 6 describes a tenfold increase in relative level of $p34^{cdc2}$-like protein in suspension culture cells and excised pith treated with auxin, compared with hormone-free medium. It is likely that the changes in level follow changes in trancription since auxin-induced increase in cdc2 mRNA has been observed in soybean root (Miao et al., 1993) and pea root (John et al., 1993b).

H1 histone kinase activity of $p34^{cdc2}$-like enzyme was measured after affinity purification of the enzyme by binding to $p13^{suc1}$ and was found to peak on entry into M phase in suspension culture cells synchronised by starvation for nitrogen, phosphorous, or sucrose and in cells synchronised by inhibition with aphidicolin (Chapter 3). The mitotic appearance of H1 histone kinase activity of $p34^{cdc2}$-like protein kinase is significant since it indicates that, (i) as in yeasts and animal cells, the cdc2 gene product may control the transition between late G2 and M phases in higher plant cell division (Nurse and Bissett, 1981; and reviewed in the Introduction) (ii) the enzyme may also be responsible in plant cells for the phosphorylation of histone H1 and lamins that is believed in yeast and animal cells to trigger chromosome condensation and nuclear envelope breakdown (reviewed by Nurse, 1990) and in the Introduction. Phosphorylation of histone H1 is required for chromosome condensation since several sites in two basic arms of histone H1, which are thought to embrace the DNA as it enters

and exits the nucleosome, become phosphorylated as cells enter mitosis and these sites are specifically phosphorylated by the $p34^{cdc2}$ protein kinase (Langan et al., 1989). The phosphorylation of histone H1 is increased about six-fold in late G2 to M phase and then rapidly declines as the nuclei return to G1 phase (Bradbury et al., 1974). Dephosphorylated H1 histone was an effective substrate for $p34^{cdc2}$ isolated in late G2 to M phase in yeast and human cells and it was found in this study to be equally effective as the substrate of $p34^{cdc2}$-like protein kinase from *N. plumbaginifolia.*

An α-casein activity of $p34^{cdc2}$-like protein kinase could be detected in transition from late G1 to S phase in synchronous suspension culture cells resuming after starvation for nitrogen. This finding resembles an observation of casein kinase activity in G1 phase immunopreciptates of $p34^{cdc2}$ in yeast (Simanis and Nurse, 1986) and it raises the possibility that, as in fission yeast, there may be G1/S active forms of $p34^{cdc2}$-like protein kinase in the higher plant cell division cycle. This is the first indication of such activity from a plant cell. However the identity of the enzyme responsible for this activity cannot be concluded with certainty because casein cannot be used to preferentially detect a G1/S type of activity of $p34^{cdc2}$, since casein is clearly phosphorylated by the mitotic form of the cdc2 kinase from yeast (Simanis and Nurse, 1986) and human cells (Draetta and Beach, 1988). Comparison with mammalian cells and the temporal correlation with S phase raises the possibility that α-casein kinase activity at late G1 phase in plant cells could be in part due to a different but closely related variant of cdc2, termed CDK2, which has been characterised in animal cells. CDK2 complexes with cyclin E at late G1 and with cyclin A during S phase and is preferentially catalytically active at these times. In the work described in this thesis the purification of the enzyme by $p13^{suc1}$ beads would also be expected to recover CDK2, if it is present in plants, so its activity could have been detected in these experiments. A definitive investigation will be possible when reagents become available for detecting plant G1 cyclins and cyclin A proteins and when the natural substrates of G1 active forms of $p34^{cdc2}$ kinase are established, but this is not currently possible and it has not yet been established whether plants have G1 cyclins..

7.2. Significance of protein phosphorylation at plant mitosis

Consistent with the importance of protein phosphorylation at mitosis, which is implied by the peak of $p34^{cdc2}$-like kinase activity at this time, cells could be blocked in mitosis by disturbance of protein phosphorylation levels with okadaic acid, an inhibitor of type 1 and type 2A protein phsphorylation. When plant cells were inhibited with 12 μ M of okadaic acid they arrested with G2 levels of DNA in early prophase. When inhibited with 20 μM of okadaic acid chromosomes showed abnormal more highly condensed configurations including both granular and fragmented forms of chromatin. Cells treated with 12 μM okadaic acid and control uninhibited cells both activated $p34^{cdc2}$-like protein kinase at the same time, although catalytic activities were higher in the treated cells. They also disassembled PPBs at the same time although the treated cells had not reached metaphase. This evidence of continued $p34^{cdc2}$-like kinase activation and continued PPB breakdown, together with reports that monoclonal antibody specific for PSTAIR peptide could react in PPB microtubules with a putative protein kinase related to $p34^{cdc2}$ (Mineyuki et al., 1991) and that microinjection of $p34^{cdc2}$ causes depolymerisation of the cytoplasmic microtubules in animal cells (Lamb et al., 1990), raises the possibility that preprophase band disassembly could be triggered by $p34^{cdc2}$-like kinase activation and that $p34^{cdc2}$-like kinase activity could be a common signal for spindle formation and nuclear envelope breakdown (John et al., 1993a). Certainly these last events, which are blocked by okadaic acid, are not necessary precursors of PPB breakdown. The hypothesis, that PPB microtubule disassembly could be triggered by increase in $p34^{cdc2}$-like protein kinase activity, has recently been further supported by the observation that PPB breakdown can be inhibited by staurosporine, which is a protein kinase inhibitor that inhibits $p34^{cdc2}$ derived from animal cells, therefore indicating a requirement for protein kinase activity for PPB breakdown (Katsuta and Shibaoka, 1992).

7.3. $p34^{cdc2}$ and cell differentiation or dedifferentiation in higher plants

The control of cell division in higher plant tissues must achieve not only a balance of division with the growth, which is necessary to maintain cell size and may be achieved in a similar way to that in yeast cells (John, 1984), but also a cessation of division in cells that leave the meristem. Cessation of division activity that occurs in actively growing and enlarging cells when leaving the meristem has no parallel in unicellular eukaryotes. Cells leaving the meristem often exceed a size that would allow division but their absention is necessary for the differentiation of specialised cell structure, which allows the development of functions such as photosynthesis, storage, transport or support. The process of abstaining from division may be regulated by $p34^{cdc2}$-like protein kinase level, as indicated by the early observations in wheat leaf and pea cotyledon, in which the level of $p34^{cdc2}$-like protein in differentiated wheat leaf cells was found to be one sixteenth of that in meristematic cells (John et al, 1991), and the level of $p34^{cdc2}$-like protein in pea cotyledon was highest on the first day after germination of and then rapidly falls as the cotyledons expand, declining by the fourth day to 6% (Gorst et al., 1991).

The decline in level of $p34^{cdc2}$-like protein in cells switching off division and switching on differentiation might be an inevitable occurence in all cells that have ceased dividing, alternatively the changes in level of $p34^{cdc2}$-like protein kinase may be part of the mechanism of differentiation and possibly a controlling element. The evidence present in this thesis that the level of $p34^{cdc2}$-like protein in long-term suspension cells did not decline when cell division was stopped by either nutrient starvation or aphidicolin inhibition (Chapter 3) was not consistent with the enzyme being unstable in non-dividing cells and therefore supported the possibility that the level of $p34^{cdc2}$-like protein kinase does play a role in switching between cell division and differentiation in higher plants (John et al., 1993a). Further evidence has come from the capacity of plant cells to specifically eliminate $p34^{cdc2}$ when absence of extensive growth means that the enzyme level cannot be depressed simply by dilution. In pea root cells that have already elongated a rapid breakdown of $p34^{cdc2}$-like protein that has been induced by auxin can

be caused by raised level of cytokinin (Johan et al., 1993a, b). These observations indicate that levels of p34^{cdc2}-like protein are under tight control.

Exit from proliferation is important for organised tissue formation since disorganised callus is characterised by the division of all cells, whereas in normal plant tissue cell division is restricted to meristems and cells leaving the meristem are able to enlarge beyond the size that could trigger division. As discussed above, the mechanism of division arrest appears to include the establishment of low levels of p34^{cdc2}-like protein, since this has been observed to correlate with developmentally-programmed exit from cell division in cells of leaf, stem and root tissue (John et al., 1990; Gorst et al., 1991; reviewed by John et al., 1993a and b). It is possible that high levels of p34^{cdc2}-like kinase may limit the capacity of cells to exit from the cell cycle and to differentiate. Evidence consistent with this possibility was observed from the level and activity of p34^{cdc2}-like protein kinase in long-term and short-term suspension cultures of *N. plumbaginifolia*. Short-term cultures, which still retained both PPB microtubules and the ability to differentiate into shoot and root tissues had normal levels of p34^{cdc2}-like protein and enzyme activity. However a long-term suspension culture had lost normal control of the enzyme and had four times the normal amount of enzyme protein and three times the normal activity. This is consistent with the possibility that establishment of low p34^{cdc2}-like protein levels is essential for the restriction of cell division to specialised meristems. Additionally there is the possibility that the raised levels of p34^{cdc2}-like protein kinase activity are at least part of the reason for the absence of preprophase bands from the long-term suspension culture cells. If, as we have suggested that p34^{cdc2}-like protein kinase activity triggers preprophase band disassembly (Chapter 4), then a raised basal level of the kinase could maintain conditions for PPB disassembly throughout the cell cycle because the basal level of the kinase activity in long-term synchronous culture cells is higher than the peak level of kinase activity that is proposed to be responsible for the disassembly of PPB in the normal cell cycle.

Resumption of cell division from differentiated cells provides a useful test of the involvement of p34^{cdc2}-like protein kinase in development transition between cell division and differentiation and in the resumption division by dedifferentiation. The level

of p34^{cdc2}-like protein in excised tobacco stem pith that was induced by either NAA alone or by both NAA and BAP was more than 10 times higher than that in the same pith at time of excision or when treated with BAP alone or when cultured in hormone-free medium. High H1 histone kinase activity of p34^{cdc2}-like protein occurred only in stem pith induced by both NAA and BAP, and in which resumption of cell division occurred. No p34^{cdc2}-like protein kinase activity could be detected in pith in which no resumption of cell division occurred. This evidence not only indicated the dual requirement for both auxin and cytokinin to induce dedifferentiation but also showed that increase in p34^{cdc2}-like protein kinase participated in the resumption of cell division in differentiated cells.

It is possible that cdc2 genes are selectively expressed in specific regions of the root and shoot apex as part of a mechanism that localises cell proliferation, for example, in wheat leaf and pea root high levels of p34^{cdc2}-like protein were restricted to the meristem (John et al., 1990, 1993a) and in *Arabidopsis thaliana* root cdc2 transcripts accumulated in the meristematic region and adjacent daughter cells but not in the quiescent center (Martinez et al., 1992). It is also possible that different members of cdc2 are involved in the control of cell division in different meristems. For example, two soybean p34^{cdc2} protein kinase genes that both can rescue the *CDC28* mutation in *S. cerevisiae* have recently been found in soybean and the relative expression levels of two soybean p34^{cdc2} genes varies in different tissues, with expression of cdc2-s5 being higher in roots and root nodules whereas the cdc2-s6 is more actively expressed in aerial tissue (Miao et al., 1993).

7.4. Phytohormone depedent control points in the cell cycle

The amounts of p34^{cdc2}-like protein that were induced by either 2,4-D-only or by 2,4-D together with kinetin in suspension culture cells, and similarly in pith cells induced by either NAA-only or by both NAA and BAP, were more than tenfold higher than in uninduced cells. Treatment with kinetin only or with BAP-only did not induce accumulation of p34^{cdc2}-like protein in suspension culture or pith cells. This evidence strengthes the conclusion that auxin is involved in the initiation of synthesis or accumulation of p34^{cdc2}-like protein kinase as observed in carrot cotyledon and pea root

tissue (Gorst et al., 1991; John et al., 1993a). This hypothesis can be supported by the recent observation that α-NAA at 0.1-0.5 μg/μl could preferentially increase the expression of cdc2-s5 *gene* in soybean root (Miao et al., 1993). However, it can be supposed that there are many steps, during the initiation of synthesis, accumulation and activation of $p34^{cdc2}$-like protein kinase, at which auxin stimulation might occur and a complete analysis is not available for any plant tissue. In pea root tissue an increase in cdc2 mRNA can be detected within 10 min of auxin treatment and suggests that stimulation of transcription is an early event (John et al., 1993b), The effects of hormone are tissue specific in the sense that high cytokinin may stimulate shoot cell proliferation but have the reverse effect in roots. In both tissues $p34^{cdc2}$ is likely to be key catalyst of the division cycle and to ensure the transcription of the gene under the different hormonal balances in root and shoot may require the presence of root and shoot specific cdc2 genes with specialised promoter regions having hormone responses tailored to the range of hormone levels in the tissue. For example the root homologue could have a higher optimum concentration of auxin for maximum rate of transcription. This is an area for future study.

High H1 histone kinase activity of $p34^{cdc2}$-like protein could be measured in pith parenchyma cells only when they were stimulated with both auxin and cytokinin. Similarly, in suspension culture cells treated with 2,4-D alone no high $p34^{cdc2}$-like protein kinase activity could be detected although a high level of the enzyme was induced, but interestingly a high activity developed rapidly when the same cells were supplemented with kinetin. This evidence consistently implies that in shoot derived cells cytokinin was necessary for catalytic activation of $p34^{cdc2}$-like kinase although cytokinin alone was unable to induce accumulation of the enzyme protein.

Although the detailed effects of auxin and cytokinin on the activity of $p34^{cdc2}$-like protein kinase are unknown there is a possibility that activation of the kinase may have correlated with changes in its phosphorylation state. This possibility is supported by evidence that a slower-migrating band of PSTAIR-containing protein (Figs. 6. 10, 6. 21), which is probably a phosphorylated form of $p34^{cdc2}$, as has been detected in oocytes, HeLa cells (Draetta and Beach, 1988) and in *Chlamydomonas* (John et al.,

1989) was found in tobacco cell extracts. The slower-migrating form was least abundant in extracts of pith cells that were not hormone stimulated or stimulated with BAP only. Both of these cell types are not cycling and the low abundance of slower migrating enzyme may indicate a predominance of an unphosphorylated inactive form of $p34^{cdc2}$ that is characteristic of early G1 phase or G0 (non-cycling) yeast and animal cells. However, the slower-migrating form predominated in extracts from both NAA-only and NAA plus BAP stimulated pith and this complicates the attribution of changes in activity to phosphorylation state since only enzyme from the NAA plus BAP treatment was fully active. This evidence is not inconsistent with the possibility of activity control by phosphorylation since in animal cells phosphorylated enzyme may be inactive if phosphorylated at two threonines and a tyrosine (hyperphosphrylated) or active if partially dephosphorylated (retaining phosphate at only one threonine) (Norbury and Nurse, 1992). Direct evidence that $p34^{cdc2}$-like enzyme from cells that are arrested in G2 phase for lack of cytokinin can be activated by reduction of its phosphorylation level has come from incubation of the enzyme with acid phosphatase *in vitro*. More than twofold higher H1 histone kinase activity could be measured when the enzyme derived from suspension culture cells that were arrested in G2 phase by lack of cytokinin supply was treated with acid phosphatase. A higher activation may not be obtainable with this enzyme since activation of $p34^{cdc2}$ protein kinase requires removal of phosphate from Thr 14 and Tyr 15 while phosphate on Thr 161/167 is maintained. The enzyme that *in vivo* is responsible for activating $p34^{cdc2}$ is cdc25, which is a specialised phosphoprotein phosphatase that recognises $p34^{cdc2}$ and removes phosphate from both Thr 14 and Tyr 15 (Millar and Russell, 1992). The $p80^{cdc25}$ enzyme is not available as an experimental reagent and the acid phosphatase that was used instead is non specific in action. Activation of $p34^{cdc2}$ by it required chance removal of both Thr 14 Tyr 15 phosphatase leaving Thr 161/167 phosphate in place, therefore presence of the activated form of $p34^{cdc2}$-like protein kinase could only be partial and transient. The *in vitro* activation does suggest that cytokinin in some way favours the dephosphorylation and activation of $p34^{cdc2}$ at initiation of mitosis.

At this early stage of analysis of plant cell cycle control points it should be remembered that the accumulation of cyclin protein (Draetta et al., 1989) may also be a factor in the activation of plant $p34^{cdc2}$. The possibility that accumulation of cyclins may be influenced by photohormones has not yet been investigated. The precise molecular mechanism by which cytokinin contributes to the activation of $p34^{cdc2}$-like protein kinase in plant cells is an interesting area for future research. The cytokinins zeatin and BAP have been reported to activate a protein kinase that is associated with chromatin and it has been proposed that nuclear protein kinases could provide molecular targets for the action of cytokinins (Selivankina et al, 1988). It is not clear from this study how directly the hormones acted on the protein kinase. If cytokinins can influence enzyme activity directly then one possibility is that cytokinins might activate a phosphoprotein phosphatase, which could activate $p34^{cdc2}$ kinase at mitotic initiation.

The results presented here lead to the conclusion that auxin stimulates the synthesis and/or accumulation of $p34^{cdc2}$-like protein kinase and that the additional presence of cytokinin is required for the activation of $p34^{cdc2}$-like protein kinase with respect to the mitotic substrate H1 histone. These requirements may explain why auxin is required for the transition from late G1 to S phase, why both auxin and cytokinin are required for the transition between late G2 and M phases in suspension culture cells of *N. plumbaginifolia*, and they help to explain the classical observation that both auxin and cytokinin are required for the resumption of cell division in differentiated tobacco pith cells.

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